CHARACTERISATION OF WOODLAND RHIZOSPHERIC ISOLATES OF *BURKHOLDERIA*

BY

CHUN-HAN CHAN

A thesis submitted for the degree of Doctor of Philosophy of the University of London and for the Diploma of Membership of Imperial College

May 2003
Department of Biology
Imperial College
London
SW7 2AZ

ABSTRACT

Throughout the decades, the genus *Burkholderia* has been an object of interest and has been under intense taxonomic examination. Due its diversity of characteristics it has proven itself to be a complex genus to categorise. The genus *Burkholderia* is comprised of pathogens or opportunistic pathogens of plants and humans; it also contains species that have bioremedial and biocontrol properties. The species contained within this genus can be found ubiquitously in the environment, in soil, water and the rhizosphere of plants. Due to its complex genome, polyphasic taxonomic methods have been utilised to categorise this genus into genomovars. Through these methods a comprehensive phylogeny between the species could be observed, helping to reveal common traits within the clades formed.

Should *Burkholderia* isolates be used for bioremediation or biocontrol, a comprehensive taxonomic grouping would be necessary to evaluate the risks to human and plant health. Novel woodland isolates of *Burkholderia* have been sampled from the environment to substantiate any species or habitat-specificity that may exist within this genus. The novel woodland isolates are to be characterised both phenotypically and genotypically. The data gained is then compared to fully characterised species within the genus *Burkholderia*.

Through the use of *recA* gene PCR analysis as a form of categorisation, there are currently 9 genomovars contained within the *Burkholderia* genus. *RecA* genomovar allocation has not been given to some species of *Burkholderia*, as well as the novel woodland isolates. It is hoped that through *recA* gene PCR analysis a tentative genomovar status can be nominated for some *Burkholderia* and novel woodland species.

ACKNOWLEDGMENTS

I would like to thank the following people:

My PhD supervisor, Dr Robert Coutts for his advice throughout my PhD studies.

Dr David Stead, Dr John Elphinstone, Dr Sean Simpkins, Neil Smith and other members of the Plant Health Group at Central Sciences Laboratory, York for their help and guidance with the taxonomic portion of this study.

Professor Tony Cass and members of his research group for use of the plate-reader.

Biotechnology and Biological Sciences Research Council (BBSRC) and Department of Environment Farming and Rural Affairs (DEFRA) for the funding of this PhD study.

Everyone whose equipment and chemicals I have borrowed during my studies.

I would like to thank my colleagues in the laboratory, all my friends especially Andrea, Caroline, Damiano, Hannah, Steve, Jill and Roger for making my time in London more memorable and fun. All the late nights writing reports and this thesis were made more bearable with you guys around me. Thanks!

Finally, I would thank Mum, Dad and my brother Chun-Sun for giving me the drive to complete this PhD. I thank them from the bottom of my heart for their faith me and their constant encouragement. I dedicate this thesis to my family.

CONTENTS

TITLE PAGE 1

ABSTRACT 2

ACKNOWLEDGEMENTS 3

CONTENTS 4

ABBREVIATIONS 15

LIST OF FIGURES 18

LIST OF TABLES 22

CHAPTER 1 INTRODUCTION 24

1.0 Background 24

1.1 Phenotypic characteristics of *Burkholderia* isolates 26

1.2 The *Burkholderia* genome 26

1.3 Clinical presentation of *Burkholderia* cepacia infection in humans 27

1.3.1 Cystic fibrosis (CF) patients 27

1.3.2 Non-cystic fibrosis patients 28

1.3.3 Transmission of *B. cepacia* between patients 29

1.4 Identifying the human pathogenic strains 29

1.5 *Burkholderia* cepacia as a plant pathogen 30

1.6 *Burkholderia* cepacia as a biocontrol and bioremediation agent 31

1.6.1 *Burkholderia* isolates as biocontrol agents 32

1.6.2 *Burkholderia* isolates as bioremediation agents 33

1.7 Nitrogen-fixation in *Burkholderia* 33

1.8 Virulence of *Burkholderia* cepacia 34

1.8.1 Type III secretion systems 36

1.8.2 Pathogenicity islands 36

1.9 Cable A gene in *Burkholderia cepacia* 37

1.10 Polyphasic taxonomy of the *Burkholderia* genus 38

1.11 Amplified fragment length polymorphism 40

1.12 16S ribosomal DNA gene sequencing 42

1.13 *RecA* gene sequencing 42

1.14 DNA-DNA hybridisation 43

1.15 Aims and objectives 44

1.16 Hypothesis for the study 45

CHAPTER 2 MATERIALS AND METHODS 46

2.0 Background 46

2.1 Isolation of novel environmental isolates of *Burkholderia* -Species-specificity **46**

2.1.1 Gram stain **47**

2.1.2 Cytochrome oxidase test **47**

2.2 *Burkholderia*-specific polymerase chain reaction (PCR) amplification **47**

2.2.1 Total DNA extracts by alkali lysis **48**

2.2.2 *Burkholderia* specific PCR amplification **48**

2.2.3 Electrophoresis of PCR products **48**

2.3 Phenotypic characterisation of novel *Burkholderia* isolates **48**

2.4 Fatty acid profiling **49**

2.5 Growth of environmental *Burkholderia* isolates at 28°C and 37°C **50**

2.6 Onion pathogenicity test **50**

2.7 Carbohydrate utilisation of *Burkholderia* species **51**

2.8 Whole cell protein profiling of *Burkholderia* species- SDS PAGE **51**

2.8.1 Protein extraction from Gram negative bacteria **52**

2.8.2 Separation of proteins *via* SDS PAGE electrophoresis **52**

2.9 Genotypic characterisation of novel *Burkholderia* isolates **52**

2.10 BOX element (BOX) and enterobacterial repetitive intergenic consensus (ERIC) PCR analysis **53**

2.10.1 Isolation of genomic DNA from *Burkholderia* species **53**

2.10.1 BOX PCR amplification **53**

2.10.3 ERIC PCR amplification **53**

2.10.4 Electrophoresis of fingerprinting PCR products (BOX and ERIC) 54

2.11 Phylogenetic comparison of gel patterns by GelCompar computer analysis 54

2.12 Amplified fragment length polymorphism (AFLP) analysis 56

2.12.1 Modification of template DNA by double restriction digests 57

2.12.2 Preselective PCR amplification of fragments with *Eco*RI and *Mse*I adaptors 57

2.12.3 Selective PCR amplification of *Eco*RI and *Mse*I modified fragments 57

2.12.4 Acrylamide gel electrophoresis of AFLP amplification products 58

2.13 Sequencing of the 16S rDNA gene 58

2.13.1 Purification of PCR products with PEG solutions 59

2.14 Restriction fragment length polymorphism (RFLP) patterns of 16S rDNA PCR products 60

2.15 *RecA* gene PCR analysis 60

2.15.1 *RecA* gene PCR amplification 61

2.16 Species-specific *recA* PCR analysis for *B. fungorum* and *B. sacchari* 62

2.17 Transfer of DNA onto nitrocellulose membrane *via* Southern blotting 63

2.18 DNA-DNA hybridisation of the P^{32} labelled *recA* gene of J2315 to *recA* PCR products of novel *Burkholderia* isolates 63

2.18.1 Pre-hybridisation of the DNA blot 63
2.18.2 Preparation of the oligoprobe and hybridisation process 63

2.19 Potential nitrogen fixation in novel isolates *nifK* PCR amplification 64

2.20 Agarose gel extraction 65

2.21 Preparation of competent JM109 cells using $CaCl_2$ treatment 65

2.22 Ligation of PCR DNA into pGEM®-T Easy cloning vector 65

2.23 Transformation of ligated DNA into competent JM109 cells 66

2.24 Small scale plasmid preparations 66

2.25 Analysis of cloned DNA by restriction digest and electrophoresis 66

2.26 BLASTN and BLASTX sequence analysis of cloned fragments 67

2.27 Alignment of DNA sequence data using ClustaX and GeneDoc 67

2.28 DNA-DNA hybridisation *via* the microtitre plate method 72
2.28.1 DNA fixation onto microtitre plates 72
2.28.2 Labelling probe DNA with photobiotin 72
2.28.3 DNA-DNA hybridisation / binding assay 72

2.29 Bioremediative potential of the novel *Burkholderia* isolates 73

2.30 Bioremediation of polyaromatic hydrocarbons (PAHs) pyrene and phenanthrene by novel *Burkholderia* isolates 73

2.31 PAH mix 9, pyrene and phenanthrene agar enrichment plates 74

2.32 Liquid enrichment cultures using pyrene and phenanthrene 75

CHAPTER 3 ISOLATION AND PHENOTYPIC CHARACTERISATION OF NOVEL *BURKHOLDERIA* ISOLATES 77

3.0 Background 77

3.1 Environmental sampling –Autumn 1999, Sand Hutton, York, UK 77

3.1.1 Description of sampling areas 78

3.2.1 Area 1 – Bramble woodland, Sand Hutton, York, UK 79

3.2.2 Area 2 – Open woodland, Sand Hutton, York, UK 79

3.1.2 *Burkholderia*-specific PCR analysis from Autumn 1999, environmental sampling, York, UK 79

3.2 Environmental sampling – Spring 2000, farmland / agricultural land, York, UK 81

3.2.1 Description of sampling area 82

3.2.1.1 Area 3 -Langlands- Barley field- clay soil 82

3.2.1.2 Area 4-Town centre- Barley field- clay soil 83

3.2.2 *Burkholderia*-specific PCR analysis of the colonies isolated – Spring 2000 83

3.3 Environmental sampling – Summer 2000, Hampstead Heath (woodlands), UK 83

3.3.1 Description of the sampling areas 84

3.3.1.1 Area 5 - Woodland area, Hampstead Heath, London, UK 84

3.3.1.2 Area 6 - Landscaped area, Kensington Gardens, London, UK **85**

3.3.2 Burkholderia-specific PCR analysis of the colonies isolated – Spring and Summer 2000 **85**

3.4 Environmental sampling -Summer 2001, Hampstead Heath, UK **85**

3.4.1 *Burkholderia*-specific PCR analysis of colonies isolated-Summer 2001 **86**

3.5 Environmental sampling- Autumn 2001, Sand Hutton, York, UK Area 5 and 6 **87**

3.5.1 Description of sample area **88**

3.5.1.1 Area 8 - Landscaped land- Central Sciences Laboratory grounds, Sand Hutton, York, UK **88**

3.6 Prevalence within environmental samples and seasonality of *Burkholderia* **89**

3.7 Fatty acid profile / FAME analysis of novel *Burkholderia* isolates **91**

3.8 Fatty acid profile analysis of novel isolates and *Burkholderia* type strains **95**

3.9 Comparative study of fatty acid profiles at time 24 and 48h **95**

3.10 Growth of novel environmental isolates at 28°C and 37°C **98**

3.11 Onion pathogenicity test of novel *Burkholderia* isolates **100**

3.12 Carbohydrate utilisation – API 20NE and API 50CH 101

3.13 Protein profiling of *Burkholderia* species using SDS PAGE gel electrophoresis 108

3.14 DISCUSSION OF PHENOTYPIC CHARACTERISATION 111

3.14.1 Environmental sampling 111

3.14.2 *Burkholderia*-specific PCR 112

3.14.3 Fatty acid profiling 113

3.14.4 Colonial morphology of novel *Burkholderia* isolates 113

3.14.5 Carbohydrate metabolism of novel *Burkholderia* species 114

3.14.6 Protein profiles of *Burkholderia* isolates 115

3.14.7 Speciation of novel *Burkholderia* isolates 116

CHAPTER 4 GENOTYPIC CHARACTERISATION OF NOVEL *BURKHOLDERIA* ISOLATES 117

4.0 Background 117

4.1 BOX PCR analysis of novel environmental isolates of *Burkholderia* 117

4.2 Analysis of BOX PCR dendrogram of all *Burkholderia* isolates - Operator differences 120

4.3 Analysis of BOX PCR dendrogram of novel *Burkholderia* isolates 1999-2001 125

4.4 ERIC PCR analysis of novel environmental isolates of *Burkholderia* 126

4.5 ERIC analysis of novel isolates of *Burkholderia* 128

4.6 Analysis of ERIC and BOX DNA fingerprint *Burkholderia* species and novel isolates – Operator differences 129

4.7 ERIC fingerprint dendrogram of *Burkholderia* species and novel isolates 132

4.8 BOX- ERIC combined dendrogram data – Operator differences 135

4.9 Combined BOX-ERIC data dendrogram of novel *Burkholderia* isolates 135

4.10 Amplified fragment length polymorphism (AFLP) analysis of *Burkholderia* isolates 136

4.11 Cable A gene PCR amplification 140

4.12 16S rDNA gene sequencing of novel environmental isolates of *Burkholderia* 141

4.13 16S rDNA phylogentic tree 142

4.14 Random fragment length polymorphism (RFLP) of 16S rDNA PCR product from novel *Burkholderia* isolates 149

4.15 *RecA* gene phylogeny of novel isolates of *Burkholderia* 151

4.16 *RecA* gene analysis of *Burkholderia* strains and novel woodland isolates 152

4.17 *RecA* diagnostic primers for *B. fungorum* and *B. sacchari* 154

4.18 *RecA* species specificity for *B. fungorum* and *B. sacchari* 156

4.19 *RecA* phylogeny of *Burkholderia* species and novel *Burkholderia* isolates 158

4.20 Presence of the *NifK* gene in novel woodland rhizospheric *Burkholderia* isolates 161

4.21 DNA-DNA binding of whole genomic DNA of *Burkholderia* species and novel woodland isolates 162

4.22 DISCUSSION OF GENOTYPIC DATA 165

4.22.1 AFLP 166

4.22.2 Presence of the *cblA* gene 167

4.22.3 16S rDNA sequencing 168

4.22.4 *RecA* gene sequencing 170

4.22.5 DNA-DNA binding 171

4.22.6 Summary 172

CHAPTER 5 BIOREMEDIATION OF POLYAROMATIC HYDROCARBONS 174

5.0 Background 174

5.1 Bioremediation of PAH by *Burkholderia* isolates 175

5.2 DISCUSSION OF BIOREMEDIATION STUDY 178

CHAPTER 6 SUMMARY

6.0 Background 180

6.1 Taxonomy of the genus *Burkholderia* 180

6.2 Taxonomy of novel *Burkholderia* isolates 184

6.3 Origins of novel environmental isolates 187

CHAPTER 7 FUTURE WORK 189

APPENDICES 191

A1 MEDIA 191

A2 BUFFERS 197

A3 GRAM STAINING SOULTIONS AND STREAKING METHOD USED FOR OBTAINING A PURE CULTURE 199
A4 SDS-PAGE BUFFERS AND SOLUTIONS 201
A5 OTHER SOLUTIONS 204
A6 REAGENTS FOR FATTY ACID PROFILING 205
A7 ENVIRONMNETAL CODING SYSTEM FOR THE *BURKHOLDERIA* LIBRARY 207
A8 REAGENTS FOR DNA-DNA HYBRIDISATION IN MICROTITRE PLATES 208
A9 API CARBOHYDRATE CODES 210
A10 SEQUENCES OF PRIMERS USED IN PCR AMPLIFICATION 213
A11 DIAGRAMMATIC REPRESENTATION OF pGEM-T®Easy VECTOR 214
A12 BOX, ERIC AND COMBINED BOX-ERIC *BURKHOLDERIA* DENDROGRAM DATA (Jane Richardson and Chun-Han Chan) 215

REFERENCES 230

ABBREVIATIONS

A	alanine
ATP	adenosine 5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
C	cysteine
°C	degrees Celsius
CF	cystic fibrosis
d	days
D	aspartic acid
dATP	deoxyadeosine 5'-triphosphate
dCTP	deoxycystosine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotides
dTTP	deoxythymidine 5'-triphosphate
E	glutamic acid
EDTA	ethyenediaminetetraacetic acid sodium salt
F	phenylalanine
G	glycine
g	gram
GLU	glucose
H	Histidine
h	hour
I	isoleucine
IPTG	isopropyl-1-thio-β-D-galactopyranoside
K	lysine
kbp	kilo-base pairs
L	litre
LB	lauria-bertani medium
LMG	Laboritorium voor Microbiologie, Universteit Gent.
M	methionine

Continued.

Mb	megabases
mg	milligram
min	minute
ml	microlitre
mM	micromolar
N	asparagine
NA	nutrient agar
nt	nucleotides
ng	nanograms
nm	nanometres
OD	optical density
P	proline
PAH	polyaromatic hydrocarbon
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PHEN	phenanthrene
PYR	pyrene
Q	glutamine
R	arginine
rDNA	ribo-deoxynucleic acid
rpm	rotations per minute
RT	room temperature
S	serine
s	second
SDS	sodium dodecyl (lauryl) sulphate
SDW	sterile demineralised water
T	threonine
TAE	tris-acetate-EDTA
Tris	tris(hydroxymethyl)aminoethane
U	unit of enzyme activity
UV	ultraviolet

Continued.

V	valine
v/v	volume: volume
W	typtophan
w/v	weight : volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
Y	tyrosine

LIST OF FIGURES

CHAPTER 1 INTRODUCTION

1.1 A diagrammatic representation of the AFLP amplification process **41**

CHAPTER 2 MATERIAL AND METHODS

2.1 Direction of primers used in *recA* gene sequencing of *Burkholderia* isolates **62**

CHAPTER 3 ISOLATION AND PHENOTYPIC CHARACTERISATION OF NOVEL *BURKHOLDERIA* ISOLATES

3.1 *Burkholderia* specific PCR amplification, Autumn 1999, Gel 1 and 2 **80**

3.2 *Burkholderia* specific PCR amplification of environmental isolates, Summer 2001, Hampstead Heath, UK **87**

3.3 The variance of viable *Burkholderia* isolates recovery from different sample types **89**

3.4 Effects of seasonal conditions on viable *Burkholderia* recovery from the environment **90**

3.5 Protein profiling of *Burkholderia* using SDS-PAGE gel electrophoresis: Gel 1, Novel environmental *Burkholderia* isolates **108**

3.6 Protein profiling of *Burkholderia* using SDS-PAGE gel electrophoresis: Gel 2: Environmental isolates and *B. cepacia* type strain **109**

3.7 Protein profiling of *Burkholderia* using SDS-PAGE gel electrophoresis:

Gel 3: Different clinical strains of *B. cepacia* **109**

3.8 Protein profiling of *Burkholderia* using SDS-PAGE gel electrophoresis: Gel 4: Different species of *Burkholderia* **110**

CHAPTER 4 GENOTYPIC CHARACTERISATION OF NOVEL *BURKHOLDERIA ISOLATES*

4.1 BOX PCR of novel environmental isolates of *Burkholderia* **118**

4.2 BOX PCR fingerprints of *Burkholderia* species **119**

4.3 Dendrogram of BOX fingerprints from novel woodland isolates and *Burkholderia* species – Operator differences **121**

4.4 Dendrogram of BOX fingerprints from novel woodland isolates and *Burkholderia* species – Time differences **122**

4.5 Dendrogram of BOX DNA fingerprints from *Burkholderia* species **123**

4.6 ERIC DNA fingerprints of novel woodland *Burkholderia* isolates **127**

4.7 ERIC DNA fingerprints of *Burkholderia* species **128**

4.8 ERIC DNA fingerprint dendrogram of *Burkholderia* species and novel isolates **130**

4.9 BOX-ERIC combined dendrogram data from novel woodland isolates and *Burkholderia* species **133**

4.10 Dendrogram of AFLP fingerprints from 72 novel woodland isolates and

Burkholderia species **138**

4.11 Cable A PCR analysis of *B. cepacia* J2315 (LMG 16656) Edinburgh-Toronto epidemic strain and clinical strains of *B. cepacia* **140**

4.12 16S rDNA gene product of novel environmental isolates of *Burkholderia* **142**

4.13 16S rDNA phylogenetic tree of novel woodland *Burkholderia* isolates and bioremedial *Burkholderia* **143**

4.14 16S rDNA phylogenetic tree of novel woodland isolates, *Burkholderia cepacia* complex isolates and other species of *Burkholderia* **145**

4.15 16S rDNA phylogenetic tree of novel woodland isolates, *Burkholderia cepacia* complex isolates and strains of *Burkholderia* **147**

4.16 Random fragment length polymorphism (RFLP) of 16S rDNA PCR product from novel *Burkholderia* isolates Gel 1, Gel 2 and Gel 3 **150**

4.17 *RecA* gene analysis of *Burkholderia* strains and novel woodland isolates **153**

4.18 *RecA* diagnostic primers for *B. fungorum* Gel 1 and 2 **154**

4.19 *RecA* diagnostic primers for *B. sacchari* **155**

4.20 Translated *recA* gene sequence from novel isolates, *B. fungorum*, *B. sacchari* and *B. caledonica* **157**

4.21 *RecA* gene phylogeny of *Burkholderia* species and novel *Burkholderia* isolates- full sequence data **158**

4.22 *RecA* gene phylogeny of *Burkholderia* species and novel *Burkholderia* isolates- clipped sequence 800bp **160**

4.23 *NifK* gene amplification of novel isolates and *B. vietnamiensis* **161**

LIST OF TABLES

CHAPTER 2 MATERIALS AND METHODS

2.1 Description of *Burkholderia* isolates and other bacteria used in the study **55**

2.2 β-*Proteobacteriacae recA* gene sequence accession numbers used in *recA* analysis **61**

2.3 *Burkholderia* species and their origins with 16S rDNA gene sequence accession numbers **68**

CHAPTER 3 ISOLATION AND PHENOTYPIC CHARACTERISATION OF NOVEL *BURKHOLDERIA* ISOLATES

3.1 Environmental sampling- Autumn 1999 Sand Hutton, York, UK **78**

3.2 Environmental sampling- Spring 2000, farmland / agricultural land, York, UK **82**

3.3 Environmental sampling-Summer 2000, Hampstead Heath (woodlands), UK **83**

3.4 Environmental sampling- Hampstead Heath, Summer 2001, Area 5 and 6 **85**

3.5 Environmental sampling- Sand Hutton, York, UK-Autumn 2001 **88**

3.6 Fatty acid profile / FAME analysis of novel *Burkholderia* isolates and type strains **92**

3.7 Effects of an extended incubation time on the FAP ID **95**

3.8 Colony characterisation and sources of novel environmental isolates **96**

3.9 Growth of novel environmental isolates at 28°C and 37°C **99**

3.10 Onion pathogenicity test of novel *Burkholderia* isolates **100**

3.11 API 20NE carbohydrate profile of novel *Burkholderia* isolates **103**

3.12 API 50CH carbohydrate profile of novel *Burkholderia* isolates **105**

CHAPTER 4 GENOTYPIC CHARACTERISATION OF NOVEL *BURKHOLDERIA* ISOLATES

4.1 Binding values of whole genomic DNA from *Burkholderia* species and novel woodland isolates- Microtitre format **164**

CHAPTER 5 BIOREMDIATION OF POLYAROMATIC HYDROCRABONS

5.1 Level of growth of *Burkholderia* isolates on MM2 medium supplemented with PAH and PAH & glucose **176**

Paul Cook

05/04/2005 12:05

To: Joanne Aish/MSN/FSA@FSA
cc: Judith Hilton/MSN/FSA@FSA, Geraldine Hoad/MSN/FSA@FSA
Subject: Salmonella update

Jo

Linda Ward rang me this morning with an update on the *Salmonella* Virchow figures.

This year LEP have had 9 isolates of *S*.Virchow PT8 with the antimicrobial resistance profile of the outbreak strain reported in the CDR a few weeks ago. Of these 8 were from the Oldham cluster which we were already aware of and 1 isolate reported by LEP on 14 March. This was from Northern Ireland (Antrim hospital lab) for week 8. I have asked Linda to check whether there is any information about foreign travel associated with this case. LEP have had 14 other *S*.Virchow PT8 isolates this year although these don't have the antimicrobial resistance profile of the outbreak strain.

In relation to the *Salmonella* Panama from cooked chicken mentioned at this morning's update meeting, Linda said that they have had 4-5 isolates from humans so far this year, all sporadics from England. She would normally expect this number for the time period. LEP will compare the cooked chicken isolates to the human ones.

In catching up with my E-Mails I see that the cooked chicken which tested positive for *Salmonella* Panama was from a consignment on a ship from Thailand (manufacturer TH49) rejected by the BIP in Northern Ireland and a RASFF was forwarded to the Commission. The samples were also positive from *Citrobacter.* The consignment was destroyed although I think we should consider testing other consignments of chicken from this manufacturer. The importer is Universal Meats (UK) Ltd and is based in Orpington, Kent - can you liaise with the food incident team on this please.

Thanks

Paul



Donna Stephenson-Baah
04/04/2005 18:11

To: Joanne Aish/MSN/FSA@FSA, Geraldine Hoad/MSN/FSA@FSA, Paul Cook/MSN/FSA@FSA
cc: Ann Reason/LAES/FSA@FSA
Subject: Re: Longday Chicken

Jo

I refer to the **negative** sample results (copied to you) received last week from Kings Lynn. Based upon the results, can you please advise where we go from here? What feedback, if any, should the Agency give to KL & Longday chicken?

Regards, Donna

CHAPTER 1

INTRODUCTION

1.0 Background

The genus *Pseudomonas* was found to be a highly heterogeneous group and was distributed into five ribosomal RNA (rRNA) groups as a result of rRNA-DNA hybridisation studies (Palleroni *et al.*, 1973). *Pseudomonas* homology group II of this genus comprised of pathogens or opportunistic pathogens of plants and animals (Palleroni, 1993). Contained within this group were *Pseudomonas caryophylli*, *Pseudomonas cepacia*, *Pseudomonas gladioli*, *Pseudomonas mallei* and *Pseudomonas pseudomallei*. In 1992, *Pseudomonas* rRNA group II was reclassified through the use of the 16S rDNA gene sequence phylogeny (Yabuuchi *et al.*, 1992). The formation of a new clade of organisms distinct from the rest of the *Pseudomonas* species was found within a 16s rDNA gene tree. After further characterisation the novel genus nomination *Burkholderia* was given, named after the plant pathologist Walter Burkholder who first described *Pseudomonas cepacia* as the causative agent of sour rot in onions in 1949 (Burkholder, 1950, Ballard *et al.,* 1970).

It has been previously thought that *Burkholderia cepacia* was a single species, but through the improvement of taxonomic methods it was found to be a highly heterogeneous group containing several different species. The *B. cepacia* complex (Bcc) consists of at least nine species or genomovars which are phylogenetically closely related (Coenye *et al.,* 2001d; 2001e). The term genomovar is used to describe phenotypically similar by genotypically distinct groups of bacterial strains. The species contained within the *B. cepacia* complex are *B. cepacia* (genomovar I), *Burkholderia multivorans* (genomovar II), *B. cepacia* (genomovar III), *Burkholderia stabilis* (genomovar IV), *Burkholderia vietnamiensis* (genomovar V), *B. cepacia* (genomovar VI), *Burkholderia ambifaria* (genomovar VII), *Burkholderia anthina* (genonmovar VIII) and *Burkholderia pyrrocinia* (genonmovar IX) (Coenye *et al.*, 2001d, 2001e, Vandamme *et al.*, 2002b).

B. cepacia complex isolates can be found ubiquitously in the environment in the soil, water and on plant surfaces (Yohalem & Lorbeer 1994). *B. cepacia* is a Gram negative rod (1.6 - 3.2 X 0.8 - 1.0 μm in size) and motile through polar flagella. It is an obligate aerobe with optimum growth ranging between 30-35°C (maximum 42°C) for

environmental strains and 37°C for clinical strains (Stainer *et al.*, 1966; Ballard *et al.*, 1970). On nutrient agar colonies appear to be cream in colour, dome shaped and of approximately 1mm in diameter. However, some strains of *B. cepacia* complex produce a non-fluorescent yellow / green pigment; other pigmentations have also been known to occur (Schwartz & Mohan, 1965). Between two and four replicons (chromosomes) have been found in clinical isolates of *B. cepacia* with a genome size ranging from 5.0 to 7.7Mbp (Wigley & Burton, 2000). A genome sequencing project at the Sanger Institute, UK has revealed that clinical isolate *B. cepacia* strain J2315 has a whole genome size of 8.056Mb made of three chromosomes sized 3.870, 3.217 and 0.876Mb. Along with the three chromosomes a plasmid sized at 92.7kb was found. The total genome G+C content for B. cepacia J2315 was 66.9%. Non-clinical isolates of *B. cepacia* have been shown to have two or three large replicons with a total genome size ranging from 4.7 to 7.8 Mbp (Wigley & Burton, 2000). For example, *B. fungorum* strain LB400 is currently being sequenced, it is sized at 8.1Mb comprising of three large chromosomes and one mega-plasmid. The sequencing of *B. fungorum* strain LB400 is being carried out at the Doe Joint Genome Institute (JGI). The name *B. cepacia* is usually reserved for isolates from genomovar I, which contains the original type strain. Genomovar III and VI await species designation if differential phenotypic tests can be found.

B. cepacia complex isolates can be described as a plant pathogen of onions (*Allium cepa* L.) (Burkholder, 1950; Yohalem & Lorbeer, 1994; Yohalem & Lorbeer, 1997), a biocontrol agent for maize (Hebbar *et al.*, 1992;), peas (Parke, 1990), bananas (Pan *et al.*, 1997) tomatoes and peppers (Mao *et al.*, 1998) against fungal and bacterial pathogens. It is also a bioremediation agent able to degrade complex chemical compounds (Bopp, 1986; Hayatsu *et al.,* 2000) and a human pathogen in immunocompromised humans such as cystic fibrosis (CF) patients (Goldmann & Klinger, 1986; Sajjan *et al.*, 1995; Mahenthiralingam *et al.*, 1997). It has also been reported to cause infections in chronic granulomatous disease (CGD) where the oxidative killing of micro-organisms is disabled (Winkelstein *et al.*, 2000; Speert, 2002). The micro-organisms are instead killed by non-oxidative means which *B. cepacia* complex isolates are resistant to, therefore prolonging their survival (Speert *et al.*, 1994; Speert, 2002).

Members of the genus *Burkholderia* have the ability to metabolise a wide range of organic compounds as a carbon source, thus making some members prime

candidates for use in the bioremediation of soil and groundwater contaminated with chlorinated hydrocarbons and herbicides (Bopp, 1986; Yohalem & Lorbeer, 1994).

Plant pathologists have demonstrated that *B. cepacia* is effective as a biocontrol agent against soil-borne, foliar and post-harvest diseases (Kawamoto & Lorbeer, 1974). Many strains of *B. cepacia* produce one or more types of antimicrobial agents that are active against a broad range of plant pathogens (Cain *et al.*, 2000). When used in agriculture, *Burkholderia* species may help reduce the need and use of chemical pesticides, which have been known to cause problems in the environment and in human health (Parke, 1998; Govan & Vandamme, 1998; Parke & Gurian-Sherman, 2001; Leigh *et al.*, 2002).

1.1 Phenotypic Characteristics of *Burkholderia* isolates

Burkholderia isolates are nutritionally diverse organisms enabling them to live in both soil and plant habitats (Ballard *et al.,* 1970; Yohalem & Lorbeer, 1994; Chiarini *et al.*, 2000). The phenotypic profile of *B. cepacia* is variable due to some strains being able to utilise various carbon sources and some not. *B. cepacia* is able to utilise the following carbon sources: arabinose, xylose, dextrose, levulose, lactose, maltose, sucrose, glycerol, mannitol and salicin. It is also able to utilise citric, hippuric, malonic and tartaric acids (Burkholder, 1950, Richard *et al.*, 1981; Gillls *et al.*, 1995). *Burkholderia* species have also been known to multiply in distilled water and dilute disinfectants such as cetrimide and chlorohexidine. Due to this property *Burkholderia* isolates have been able to proliferate and cause nosocomial outbreaks in hospital patients (Geiger *et al.,* 2001). Its highly varied nutritional competence makes *B. cepacia* an ideal candidate for bioremediation. Plant pathogenic strains of *B. cepacia* can produce an endopolygalacturonase at low pH which is responsible for onion tissue maceration (Ulrich, 1975).

1.2 The *Burkholderia* genome

The genome of *Burkholderia* is complex and this can be reflected through its diverse nutritional array. In previous studies it has been shown that some *Burkholderia* strains contained between 2-4 replicons (chromosomes), the genome can vary from 4-9 Mb in size (Cheng & Lessie 1994, Lessie *et al.*, 1996, Wigley & Burton, 2000, Parke & Gurian-Sherman, 2001). The presence of insertion sequence (IS) elements assisted in

the uptake and removal of genetic material by the giving it a 'genomic plasticity' (Miché *et al.*, 2001).

To gain a better understanding of the *Burkholderia* genome a representative clinical (J2315, ET12 lineage, genomovar IIIA) and *Burkholderia fungorum* strain LB400 (LMG 16225) which is found in both environmental and clinical samples were sequenced. *Burkholderia pseudomallei* strain K96243, a causative agent of melioidosis, an infection of the wounds through contaminated water or soil which commonly occurs in tropical regions, is also currently being sequenced. Through this, it is hoped that elements contributing to the pathogenicity and epidemiology of pathogenic strains of *B. cepacia* may be found. These projects are being undertaken by the Sanger Centre (UK) and DOE Joint Genome Institute (Department of Environment, UK).

1.3 Clinical presentation of a *Burkholderia cepacia* complex infection in humans

Infections in humans caused by some *Burkholderia* species occur when immunity has been lowered or compromised. This may be due to a pre-existing condition such as cystic fibrosis (CF), from a systemic infection caused by another organism or from a wound (Govan & Harris, 1985; Govan & Deretic, 1996). It is not known whether all *B. cepacia* complex isolates recovered from infections were initially the causative agents rather than transient infections (Govan & Vandamme, 1998).

1.3.1 Cystic fibrosis (CF) patients.

CF is an inherited genetic disease which results in a defect in the sodium and chloride pumps in the epithelial lung cells. The lungs then become lined with a thick mucus due to the imbalance of ions across the epithelial cells. This results with the lungs being clogged up and the patient becoming prone to lung infections due to the inadequate removal of the mucus through mucociliary movements.

The frequency of CF gene in Caucasians is 1 in 2,500 individuals. The most common cause of mortality and morbidity within CF patients is from pulmonary (lung) infections. The susceptibility of CF patients to pulmonary infections has been acknowledged since the first cases of CF were described. Death during infancy and early childhood was inevitable until the introduction of effective treatments and chemotherapy (Goldmann & Klinger, 1986; Govan & Deretic, 1996).

B. cepacia complex isolates appear to have an inherent resistance to a range of antibiotics, with the only effective antibiotics being cephalosporins (ceftazidime), quinolones (ciprofloxacin) and trimethroprim (Speert, 2002). Infections caused by *B. cepacia* can result in a 'super- infection' and critical health of 40% of CF patients which could be difficult to treat due to limited number of effective antibiotics. *B. cepacia* is not a common commensal organism, thus carriers in hospitals pose a serious health risk to CF patients and other immunocompromised patients.

In CF patients an infection caused by *B. cepacia* can present itself as a pulmonary infection which may lead to necrotising pneumonia with a fatal bacteraemia. This is known as 'Cepacia Syndrome' and has been reported in 20% of CF patients (Vandamme *et al.*, 1997; McManus *et al.*, 2003). Cepacia syndrome leads to two clinical presentations, a rapid and fatal deterioration of the patient and secondly the chronic colonisation with or without the slow deterioration of lung function (Govan & Deretic, 1996; Vandamme *et al.*, 1997). Pulmonary infections ultimately lead to 90% of the causal deaths in CF patients. Infections caused by *B. cepacia* can result in the increased rate of morbidity and mortality amongst CF patients.

The transmission of *B. cepacia* has been linked through social and medical groupings such as medical units and clinics *i.e.* contact with colonised individuals and other CF patients. *B. cepacia* genomovar III was found to cause the majority of patient-to-patient transmission in CF patients (Mahenthiralingam *et al.*, 2000a). Strains of *B. cepacia* genomovar IIIA were found to be the cause of majority of infections (Bevivino *et al.*, 2002; Mahenthiralingam *et al.*, 2002). Even though the majority of infections in CF patients are caused by genomovar III strains, infections caused by other genomovars can not be ignored. For example, *B. multivorans* has also been found to cause a high proportion of infections (Whiteford *et al.*, 1995).

1.3.2 Non-cystic fibrosis patients.

In non-CF patients, *B. cepacia* causes opportunistic infections such as endocarditis, catheter-associated urinary tract infections, wound infections *e.g.*, abscesses and burns, intravenous catheter associated bacteraemia (Holmes *et al.*, 1998) and chronic granulomatous disease (Govan *et al.*, 1996). Isolates of *B. cepacia* were also found responsible for 'foot rot' in US troops in Florida on swamp training (Taplin *et al.*, 1971). Patients suffering from chronic granulomatous disease (CGD) were found to be susceptible to infections caused by *B. cepacia* complex isolates. The infections cause

pneumonia and septicaemia within a CDG patient (Lacy *et al.*, 1993; Speert *et al.*, 1994; Speert, 2002).

1.3.3 Transmission of *B. cepacia* between patients.

Transmission between CF and non-CF patients has been associated with serious health problems / risk and death. There have been infections reported which have either been caused by *Burkholderia gladioli* or an intermediate bacterium with characteristics of *B. gladioli* and *B. cepacia* (Vandamme *et al.*, 1997).

The transmission of *B. cepacia* complex isolates within hospitals has been due to person to person contact, the contamination of intravenous solutions and disinfectant preparations (Speert, 2002). Some strains of *B. cepacia* have the ability to survive in disinfectant solutions, hence the frequent contamination of creams and ointments causing outbreaks within hospitals. *B. cepacia* is able to acquire resistance to antibiotics and other antimicrobial agents, thus making it a difficult organism to treat (Govan & Deretic, 1996). The segregation of patients with a *B. cepacia* complex infection resulted in the significant reduced transmission of the bacteria (Mahenthiralingam *et al.*, 2001).

1.4 Identifying the human pathogenic strains

There have been many methods used to distinguish between non-pathogenic and pathogenic strains from the *Burkholderia* genus both phenotypically and genotypically. Commercial systems such as API 20NE (BioMerieux) have proved themselves to be inadequate in the identification of *Burkholderia* to the species level due to variable carbohydrate utilisation profiles (LiPuma, 2000). For example, characterisation through biochemical profiles such as pectinase production, onion pathogenicity, bacteriocin production and susceptibility (Hagedorn *et al.*, 1987; Yohalem & Lorbeer, 1994) and genomic analysis using random amplified polymorphic DNA (RAPD), pulsed field gel electrophoresis (PFGE) (Mahenthiralingam *et al.*, 1997; 2002), 16S rRNA (Coenye *et al.*, 2001a, 2001b, 2001c, 2001d, Fiore *et al.*, 2001) and *recA* gene sequences (Mahenthirlingam *et al.*, 2000a) have been used to attempt to characterise the various *Burkholderia* species.

Recent studies have leaned towards genetic markers such as the cable A (*cblA*) pilin gene (Sajjan *et al.*, 1995) and the 1.4kb fragment called the *B. cepacia* epidemic strain marker (BCESM) (Clode *et al.*, 2000). The BCESM marker is not present in non-epidemic strains and therefore can be used as a form of identification (Mahenthiralingam *et al.*, 1997) There are well-characterised strains which have caused epidemic infections in CF patients *e.g.* the highly virulent Edinburgh-Toronto strain. Transmissibility and virulence is dependent on the strain, most are not involved with epidemics amongst CF patients. Greater transmissibility is known to be associated with the presence of the *cblA* pili and the BCESM in *B. cepacia* J2315 (ET12 lineage) (Parsons *et al.*, 2003). Some *B. cepacia* isolates are closely related to isolates that caused epidemics but were not known to cause human infection. The wide spread distribution of different epidemic strains of *B. cepacia* suggests that the highly transmissible strains are emerging independently and randomly (Holmes *et al.*, 1998).

1.5 *Burkholderia cepacia* as a plant pathogen

Plant pathogenic strains of *B. cepacia* cause the rotting of host tissues by the release of the pectolytic enzyme, endopolygalactronase (Ulrich, 1975). Infection of host tissue by lowering the pH to pH 4.0 produces ideal conditions for endopolygalactronase to work at an optimal level thus increasing the enzyme activity (Ulrich, 1975; Gross & Cody, 1985). Sour skin is an affliction in onions caused by some strains *B. cepacia* (genomovar I). It causes the yellowing of the onion scales, the appearance of the onion is yellow, slimy and sour smelling as compared to a healthy sweet smell (Burkholder, 1950). Onions are relatively resistant to infection by *B. cepacia* prior to bulbing and the environment does not become favourable for the bacterium until after bulbing (Kawamoto & Lorbeer, 1974). Infection starts in a wound or when the tops of the bulbs are cut off and then this becomes congested with water from rain or irrigation. The bacterium can also enter the healthy tissue when the onion tops are cut at harvest or when the foliage collapses at maturity (Schwartz & Mohan, 1965; Kawamoto & Lorbeer, 1974).

1.6 *Burkholderia cepacia* as a biocontrol and a bioremediation agent

Burkholderia species appear not to survive well in dry conditions, on dry surfaces and it has a survival period of about a week in these conditions. However, *B. cepacia* can survive quite well in water and moist conditions for many months. Therefore, the agricultural application of *Burkholderia species* will increase the human exposure to the organisms and may be a cause for concern to human and animal health (Holmes *et al.*, 1998).

Some isolates of *Burkholderia* have the ability to antagonise and repress many soil-borne pathogens. The ecological and economic benefits are enormous if the anti-fungal properties are utilised to enhance crop yields and reduce the needs for herbicides and pesticides (Hebber *et al.*, 1992; Cartwright *et al.*, 1995; Cain *et al.*, 2000). Another characteristic is the ability of some *Burkholderia* isolates to reduce / degrade complex herbicides and pesticides for bioremediation (Bopp, 1986). However, its use in agriculture raises the question of whether there is a risk of it switching from being a harmless organism into a virulent human pathogen. It has been demonstrated that virulence and resistance genes can be passed horizontally. Therefore, there is a risk that by introducing *Burkholderia* isolates into the environment alongside pre-existing soil organisms virulence and antibiotic resistance genes may be acquired at some point.

The increased use of antibiotics within intensive animal farming practices has resulted in the higher incidence of resistance genes in bacteria. In a recent study it was shown that farm manure used to fertilise soil increased the incidence of antibiotic resistance genes in the soil bacteria population (Bevivino *et al.*, 2002; Esibou *et al.*, 2002). The availability of these resistance genes in the soil is providing an ideal environment to produce a 'super-bug' which is highly resistant to antibiotics and pathogenic to plants, animals and humans.

There is a need to assess the risk to the environment and to the human population should *Burkholderia* isolates be released for agricultural use. Its ability to change its genetic make up or acquire new genes is high, therefore until more is known about the bacterium agricultural usage is not recommended.

1.6.1 *Burkholderia* isolates as biocontrol agents.

The mechanisms that make some *Burkholderia* species effective as a biocontrol agent are generally not known. However, it has been suggested that some of the biocontrol properties can be attributed to the production of antimicrobial metabolites such as antibiotics and siderophores (Imanaka *et al.*, 1965; Pan *et al.*, 1997, Lewenza & Sokol, 2001).

Biocontrol of the fungal pea pathogen *Pythium ultimum* has been achieved through the use of a specific strain of *B. cepacia. P. ultimum* causes damping off seedlings, it is a common and important disease wherever the crop is being cultivated. Some fungicides used to control this pathogen have been shown to be a risk to humans and to the environment (Parke, 1990). The risk to human health from some fungicides could suggest that *Burkholderia* isolates are potentially a safer alternative to control *Pythium ultimum*.

In a study by Hebber *et al.* (1992) of the seed inoculation of maize, rhizobacterial strain *B. cepacia* 526 was shown to colonise and multiply in the maize rhizosphere. The rapid migration rates of microbial inoculants on the roots may well be linked to high growth rates. In turn, this effect may lead to beneficial effects such as anti-fungal activity. The beneficial effects were not confined to the seed coat area but were also present in the rhizosphere and on the roots as the inoculum migrates along the roots (Hebber *et al.*, 1992).

B. cepacia was also shown to be effective in the biocontrol of the fungus *Fusarium oxysporum* f. sp *cubense*. Biocontrol of the pathogen was achieved by the bacterium colonising the surface hyphae of *F. oxysporum* f. sp *cubense.* Antifungal agents are produced by the bacterium to attack the fungus. Colonisation of the roots by *B. cepacia* also prevented infection by *F. oxysporum* f. sp *cubense*. (Pan *et al.*, 1997).

B. cepacia strain 5.5B has been found to produce two antimicrobial agents capable of controlling *Rhizoctonia* stem rot in poinsettias caused by *Rhizoctonia solani.* The antimicrobial activity was partly due to the secretion of extracellular compounds (Cain *et al.,* 2000). Experiments revealed the presence of two antimicrobial compounds called phenazine and pyrrolnitrin (Cartwright *et al.*, 1995). These two compounds can inhibit the growth of *R. solani in vitro* (Cartwright *et al.*, 1995)

1.6.2 *Burkholderia cepacia* as a bioremediation agent.

Environmental pollution can have devastating effects on flora and fauna, resulting in toxic chemicals entering the food chain. Some chemicals are known to be persistent within the environment, which highlights the need for methods of cleaning contaminated sites (Timmis & Pieper, 1999).

Due to *Burkholderia* isolates having the ability to utilise a varied range of carbon sources it has potential for the bioremediation of complex chemicals (Yabuuchi *et al.*, 1992; Gillis *et al.*, 1995; Mueller *et al.*, 1997; Hanada *et al.*, 1998; Friedrich *et al.*, 2000;). However, the use of the organism to break down complex chemical compounds should only be carried out in containers designed for this purpose until the risk to health is fully assessed. Sterilisation of the bioremediated soil also needs to be carried out to ensure the organism used to remediate the soil does not make the land useless for agricultural use where other pesticides and insecticides may be used.

B. cepacia strain PS14 has been found to be effective in the degradation of chlorinated benzenes which is a starting material in the production of insecticides, disinfectants, fungicides, herbicides, rubbers and plastics (McGowan *et al.*, 1998; Rapp, 2001; Goris *et al.*, 2002). Chlorinated benzenes are not persistent within the environment at high levels; however nano- and micromolar concentrations do persist and these levels can build up in water sources (Rapp, 2001). Enzymes which can degrade cyanide have been isolated from *B. cepacia* strain C-3 (Adjei & Ohta, 2000). This property has a great use in the mining industry and agro-processing factories as cyanide has a high toxicity. The utilisation of cyanide is possible due the limiting levels of carbon and nitrogen available for the organism (Adjei & Ohta, 2000). Key genes (the *bph* gene cassette) have been sequenced from isolates (strain LB400) possessing the ability to bioremediate polychlorinated biphenyls (PCBs) (Masters & Mohn, 2001; Nogales *et al.,* 2001; Seah *et al.*, 2001; Springael *et al.*, 2001).

1.7 Nitrogen-fixation in *Burkholderia*

The plant family *Leguminosae* has the ability to produce nodules on the roots. The nitrogen-fixing success of nodules can be attributed to a symbiotic relationship with bacteria of the genus *Rhizobium*. The presence of *Rhizobium* on the root surface results in the ability of nodules to fix atmospheric nitrogen. The capacity for nitrogen fixation has also been found in several genera of bacteria such as *Azospirilum*

brasilense, *Herbaprillium seropedicae* and *Acetobacter diazotrophicus* (Magalhães Cruz *et al.*, 2001). Their nitrogen-fixation properties help benefit the host plants by acting as biofertilizers and plant growth promoters. Nitrogen fixing bacteria can be isolates from various parts of plant stems, leaves, roots and fruits (Magalhães Cruz *et al.*, 2001).

The genus *Burkholderia* has been described as a bacterial group rich in nitrogen-fixers. However, *B. vietnamiensis* is the only species within the genus officially described as a nitrogen-fixing bacterium which has a symbiotic relationship with the plant (Gillis *et al.*, 1995; Estrada-De-Los Santos *et al.*, 2001). However, it appears that other species within this genus also possess some nitrogen-fixing properties. For example, *B. brasilenesis* (strain M130) and *B. tropicalis* (strain Ppe8) (Marin *et al.*, 2003) are two new species which have been found to fix nitrogen, as has *B. kururiensis*. Recently, *B. tuberum* and *B. phymatum* have also been shown to possess nitrogen fixing properties and were able to establish an endosymbiotic relationship within plants (Moulin *et al.*, 2001; Vandamme *et al.*, 2002a).

In study by Estrada-De Los Santos and others (2001) it was found that a high number of the nitrogen-fixing bacteria were isolated from the rhizosphere. It was suggested through 16S rDNA gene sequence phylogenetic data that other species apart from *B. vietnamiensis* may have nitrogen fixing capabilities (Estrada-De Los Santos *et al.*, 2001). The genetic characterisation of the *nifHDK* operon enabled the characterisation of nitrogen-fixing bacteria within soil and the plant rhizosphere (Minerdi *et al.*, 2001). This operon was responsible for the production of the enzyme nitrogenase. It was found that an endosymbiont *Burkholderia* isolate possessed these genes in order to assist in nitrogen fixation within the symbiotic mycorrhizal fungus *Gigaspora margarita* (Minerdi *et al.*, 2001).

1.8 Virulence of *Burkholderia cepacia*

The virulence factors involved in human, animal and plant pathogenicity are yet to be clearly defined. However, from past and recent studies it seems that species from one genomovar are recovered from infected CF patients more often than other genomovars. These virulent strains are either from an epidemic associated lineage or from genomovar III (Sajjan *et al.*, 2002).

In previous studies it has also been shown that certain virulence genes are present in both human and plant strains of *B. cepacia*. Virulence is affected by the genes involved in physiological processes for survival in host and non-host environments; these genes can be found in both pathogenic and non-pathogenic bacteria (Groisman & Ochman, 1996). These virulence genes are often thought to be confined to extrachromosomal elements; however these genes have been found on the chromosomes contained within pathogenicity islands (PAIs) (Hacker *et al.,* 1997; Hou, 1999; Karaolis *et al.*, 1999). One aspect of virulence of *Burkholderia* species is the ability to survive within macrophages and epithelial cells to demonstrate intracellular parasitism (Speert, 2002). Survival within host cells makes treatment of the infection difficult as most drugs are unable to penetrate the eukaryotic cell (Speert, 2002).

The ability of *Burkholderia* species to switch from being an environmental isolate to an opportunistic human pathogen could be due to the possession of an unusually large complex and variable genome. The genome contains numerous insertion sequences and it is divided into one to four circular replicons (Lessie *et al.*, 1996). This unusual genome may also explain the nutritional versatility and adaptability of *Burkholderia* species (Holmes *et al.*, 1998).

Virulence of a micro-organism occurs when a suitable animal/ plant host has been found. One example of virulence genes is those controlling the type III secretion system, a cluster of genes that enable the production of virulence factors which can be secreted into host cells. Chalmers & Blot (1999) indicated that the presence of insertion sequence (IS) elements allowed gene recruitment by the bacteria (Charlebois, 1999). For example, a strain of *B. multivorans* has been found to have seven IS elements within its genome (Cheng & Lessie, 1994). This promoted genetic rearrangement to improve function and survival within its immediate environment (Miché, 2001). The presence of IS elements allows genome plasticity making the bacteria more susceptible to mutagensis which is important for their adaptation (Miché, 2001). The presence of a polysaccharide gene cluster in the ET12 lineage strains and some *B. cepacia* complex members has been reported (Parsons *et al.*, 2003). The production of a polysaccharide capsule around the bacterial cell aids adherence to the host during an infection (Parsons *et al.*, 2003).

1.8.1 Type III secretion systems.

Type III secretion systems have been found in Gram-negative bacteria and they facilitate the export of virulence factors into the host cells via the plasma membrane and cytoplasm (Mecsas & Strauss, 1996; Blocker *et al.*, 2000). These virulence factors interrupt the normal functioning of the host cell to benefit the invading bacteria. Type III secretion systems are usually a characteristic of human pathogens, however some of these features can also be found amongst plant pathogens and flagellum producing bacteria. It has been speculated that type III secretion systems were acquired in order to adapt to a different host or environment. The genes responsible for the type III secretion system are usually clustered, thus making them distinguishable from the rest of the bacterial genome, the genes are usually located on PAIs (Mecsas & Strauss, 1996). The causative agent of melioidosis, *Burkholderia pseudomallei and B. cepacia* have been found to contain a type III secretion system-associated gene cluster (Winstanley *et al.*, 1999; Winstanley & Hart, 2001; Parsons *et al.*, 2003).

1.8.2 Pathogenicity islands.

PAIs contain genes that encode for one or more virulence factors such as adhesins, invasins, toxins and type III and IV protein secretion systems. The PAIs are present in pathogenic bacteria but absent from the non-pathogenic members of the same species. PAIs can occupy a large area of the bacterial genome, their sizes ranging from 10-200kb in size. The DNA sequences of PAIs usually differ from the rest of the genome due to a higher guanosine and cytosine (G+C) content (Mecsas & Strauss, 1996). PAIs are often associated with tRNA genes which can act as target sites for integration of foreign DNA; thus suggesting that they may have bacteriophage based origins. In one step a PAI can transform a benign bacterium into a virulent and pathogenic one by inserting a cluster of genes coding for virulence. Within the genome there are a large number of insertion sequences and repeated DNA motifs which help to promote genomic recombination. This has been demonstrated in some *Escherichia coli* strains (Matic *et al.*, 1996; Guttman & Dykhuizen, 1994). Little is known about the organisation of the genes and the distribution of the insertion sequence elements throughout the genome. PAIs with mobile DNA elements indicate that these sequences could be incorporated into a variety of bacterial species. PAIs can also be spontaneously deleted from the genome probably due to the sequence being unstable. However, more recent studies argue that PAIs or areas of DNA akin to PAIs can also be found within non-pathogenic bacteria (Kaper & Hacker, 1999). An example, of non pathogenic bacteria containing PAIs can be seen as large areas of

foreign DNA carrying genes for nitrogen fixation, for example in *Pseudomonas putida* and *B. vietnamiensis*. These 'symbiotic' genes are integrated into the bacterial genome, but in non-pathogenic bacteria these are more commonly known as genomic islands (Kaper & Hacker, 1999).

1.9 Cable A gene in *Burkholderia cepacia*

The cable A (*cblA*) gene is a pilin subunit structural gene which is present on the *B. cepacia* genome. The pilin protein produced by this gene is a precursor to a filamentous appendage fibre (Goldstein *et al.*, 1995; Sajjan *et al.*, 1995). The pilin aids the bacteria to adhere to the epithelial lining of the respiratory tract (Goldstein *et al.*, 1995; Sajjan *et al.*, 1995; Hales *et al.,* 1998). There is a theory that the *cblA* pilin uses the unusual environment in the CF lung to attach itself onto the mucus by using the glycoprotein carbohydrates as receptor sites (Goldstein *et al.*, 1995). At present, there are only two reports of non-CF isolates of *B. cepacia* (clinical isolate 2228-94 and a stream sediment isolate) possessing the *cblA* gene (Richardson *et al.*, 2001; Sajjan *et al.*, 2002). During a study it was found that out of all the environmental and clinical isolates tested only one genomovar III isolates contained the *cblA* gene (Bevivino *et al.*, 2002).

It has been reported in previous studies that the *cblA* gene is situated alongside other virulence genes within a PAI. Mediation of the gene transfer into the bacterial genome *via* a bacteriophage has been suggested (Karaolis *et al.*, 1999). However, any method involving the horizontal transfer of genes is also possible. The junction of the PAI and the rest of the genome may help reveal the method used to incorporate the viral DNA into the bacterial genome (Karaolis *et al.*, 1999). Transfer RNA (tRNA) genes are associated with the insertion and integration of mobile genetic elements, the factors determining the integration genes are unknown. One feature of tRNA genes is that they promote their use as an integration site for bacteriophage and other episomes (Groisman & Ochman, 1996). These tRNA genes act as some kind of genomic landmarks for the integration of transposable elements in a position-specific manner (Marschalek *et al.*, 1989). The presence of the *cblA* gene does not necessarily herald the subsequent expression of the pilin (Sajjan *et al.*, 2002). The frequency of the *cblA* gene within the environment is unknown, therefore the lack of *cblA* gene expression

needs to be addressed in order to understand virulence mechanisms (Sajjan *et al.*, 2002).

1.10 Polyphasic taxonomy of the *Burkholderia* genus

The term polyphasic taxonomy was first described by Colwell (1970) describing the taxonomy of the genus *Vibrio.* Polyphasic taxonomy aims to compile taxonomic groupings based from a combination of phenotypic, genotypic and phylogenetic data.

The genus *Burkholderia*, nominated by Yabuuchi *et al.* (1992) was formerly part of the *Pseudomonas* species ribosomal RNA (rRNA) group II which contained primarily opportunistic pathogens of plants and animals (Palleroni *et al.*, 1973). Within *B. cepacia* there are five different genomovars known as the *B. cepacia* complex (Vandamme *et al.*, 1997 and Coenye *et al.*, 1999a). There are currently nine genomovars within the genus *Burkholderia* (Coenye *et al.*, 1999b; Coenye *et al.*, 2001d). At present, the genus *Burkholderia* contains 35 validly named species, not all of these species have been allocated a genomovar status. These species being *B. andropogonis*, *B. anthina*, *B. ambifaria*, *B. brasilensis, B. caledonica*, *B. caribensis, B. caryophylli*, *B. cepacia* (type strain), *B. cenocepacia, B. cocovenenans, B. fungorum, B. gladioli, B. glathei, B. glumae, B. graminis, B.hospita, B. kirkii, B. kururiensis, B. mallei, B. multivorans, B. norimbergnesis, B. phenaziunium, B. phymatum, B. plantarii, B pseudomallei, B. pyrrocinia, B. sacchari, B. stabilis, B. thailandenis, B. terricola, B.tuberum, B. tropicalis, B. uboniae, B. vandii and B. vietnamiensis.*

The genus *Burkholderia* is continuing to develop and grow as new molecular techniques are being developed to distinguish between the various species and strains. However, there is a strict protocol to follow in establishing new species and identifying novel isolates. The criteria to fulfil in DNA–DNA hybridisation is to achieve a value greater than 70% relatedness of DNAs to indicate species similarity but this is not decisive. The use of this method should be restricted to a representative strains or a particular clade (Vandamme *et al.*, 1996). The DNA base ratio should not be more than 3% different with a species and not more than 10% for a well defined genus for G+C content. The data from 16S and 23S rRNA sequences, phenotypic features (such as carbohydrate profiles), cellular fatty acid profiles and amplified restriction site

analysis (AFLP, BOX element, REP and ERIC PCR analyses) (Hulton *et al.*, 1991; Vandamme *et al.,* 1996) should also be included in this polyphasic taxonomic system. The characterisation of the genus *Burkholderia* and other organisms has been achieved through several methods of biochemical profiling such as fatty acid profiling (FAP) (Stead, 1992) and carbohydrate utilisation pattern (Sneath *et al.*, 1981). Molecular methods such as 16S and 23S rRNA gene sequencing (Li *et al.*, 1993; O'Callaghan *et al.,* 1994; Tabacchioni *et al.*, 1995; LiPuma *et al.*, 1999; Wigley & Burton, 1999, Whitby *et al.*, 2000; Coenye *et al.*, 2001a; 2001b; 2001c; 2001d); DNA-DNA hybridisation (Ballard *et al.*, 1970; Yabuuchi *et al.*, 1992, Coenye *et al.*, 2001a, 2001b; 2001c and 2001d); pulsed field gel electrophoresis (PFGE) (Ouchi *et al.*, 1995, Wigley & Burton, 1999); random fragment length polymorphism analysis (RFLP) (Mahenthiralingam *et al.*, 1997) and amplified fragment length polymorphism analysis (AFLP) (Huys *et al.*, 1997; Janssen *et al.,* 1997; Vandamme *et al.*, 2000) *recA* gene sequencing (Mahenthiralingam *et al.,* 2000a) have all been used.

A comprehensive hierarchical system comprised of diverse *Burkholderia* isolates has been created from using both phenotypic and genotypic data to give a polyphasic taxonomic grouping (Coenye *et al.*, 2001a; 2001b, 2001c, 2001d Yabuuchi *et al.*, 1992). The phenotype of two strains may be similar but the genotype may be different, therefore, enabling group formations within a species.

In a study by Rahme *et al.* (1995), it was demonstrated that plant and human pathogens shared common virulence factors. This is due to a conservation of proteinaceous virulence factors in both plant and animal pathogens (Rahme *et al.*, 1995). This has been supported by evidence that environmental isolates in extreme circumstances could cause infection in healthy humans (Taplin *et al.,* 1971), which suggests a more adequate taxonomic grouping must be developed for this genus of bacteria.

The mechanisms of *Burkholderia* isolates ability to adapt to hostile environments is unknown, therefore research into taxonomic classification would help to explain this event. There is a need to determine the likelihood of a 'safe strain' of *Burkholderia* species being able to acquire key pathogenicity genes to become a virulent and highly pathogenic organism. This is especially important when *Burkholderia* isolates are found to be useful in the field of bioremediation and biocontrol. A comprehensive classification and prediction of possible pathogenicity may result from the use of novel

or more robust and reproducible techniques, such as 16S rDNA or *recA* gene sequencing and amplified fragment length polymorphism (AFLP).

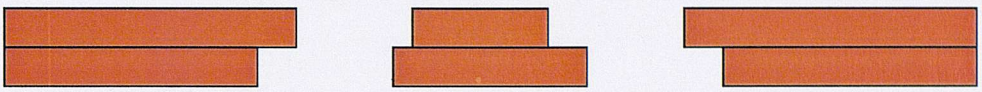
1.11 Amplified Fragment Length Polymorphism

AFLP is a method in DNA fingerprinting which was developed in by Vos *et al.* (1995). It is a combination of two molecular techniques, random fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) amplification. Whole genome DNA fingerprints are generated through the amplification of restriction fragments by PCR. The fingerprints bands produced can be modified in number by the careful selection of specific primers sets (Vos *et al.,* 1995) The selective amplification is achieved by the primers binding onto the flanking nucleotides at the end of the restriction fragments. In a typical AFLP the number of fragments generated can be anything between 50-100, these fragments are then detected on a denaturing polyacrylamide gel.

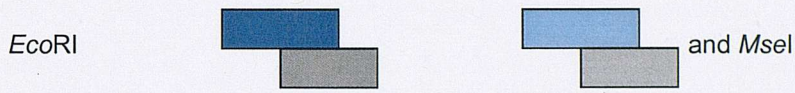
AFLP has proven itself to be a successful and highly reproducible fingerprinting technique for all types of DNA and DNAs of varying complexity. AFLP analysis is so powerful it is able to distinguish between strains of bacteria to a sub-subspecies level to determine between various pathovars (Janssen *et al.*, 1996; Clerc *et al.*, 1998). It is a rapid and highly reproducible method and is a good means of determining the taxonomic diversity and phylogenetic structure of a large selection of bacterial isolates, in this case *Burkholderia* (Coenye *et al* ., 1999b; Rademaker *et al.*, 2000).

Figure 1.1 A diagrammatic representation of the AFLP amplification process

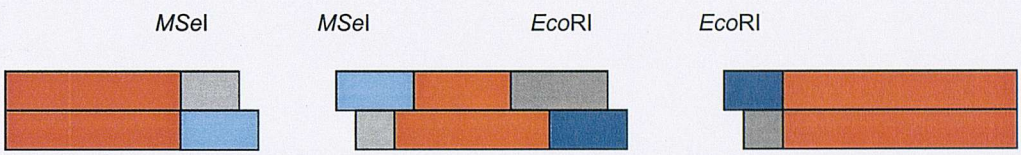
1. Digest genomic DNA with restriction enzymes *Mse*I and *Eco*RI.



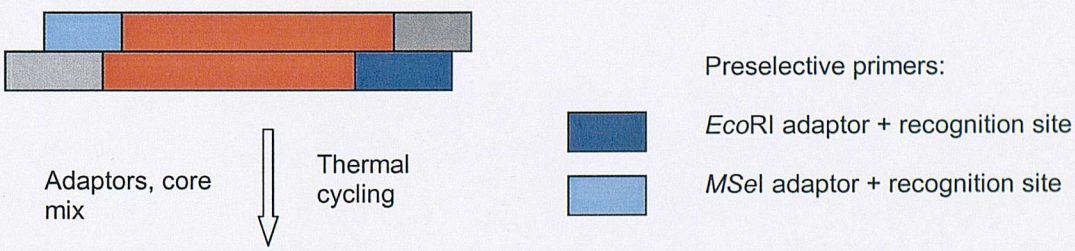
2. Ligate adaptors



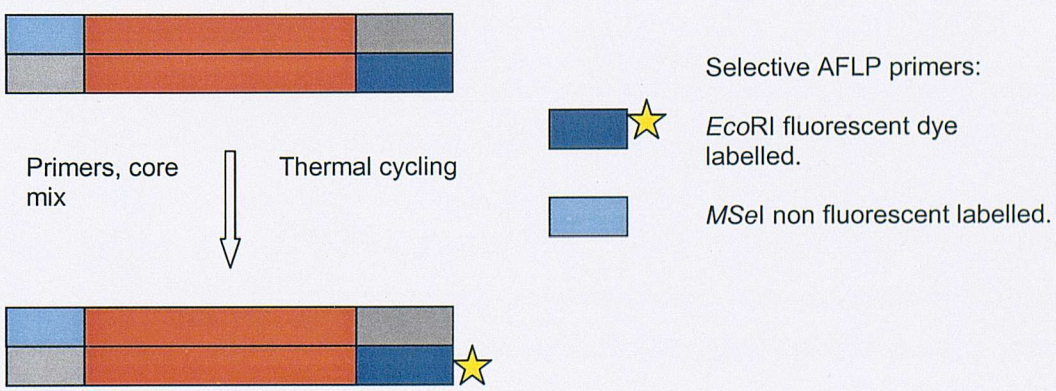
3. Modify genomic DNA fragments



Prepare DNA template with adaptors.



4. Run selective amplification.



1.12 16S ribosomal DNA sequencing

The development of molecular genetic methods during the 1980's and the 1990's revealed suitable ribosomal RNA molecules (5S, 16S and 23S rRNA) as indicators of phylogenetic relationships (Woese, 1987). The 16S rDNA gene within the bacterial genome is approximately 1500 bp in size and is proposed to be the best part of the genome to study in regard to phylogenetic relationships of bacterial species due to it being relatively conserved and possessing a slow evolutionary change rate (Woese, 1987). The variability of the gene is small within a species allowing genetic relatedness to be measured (Woese, 1987). The exclusive use of 16S rDNA sequences can not replace other taxonomic methods nor guarantee species identity.

1.13 *RecA* gene sequencing

The *recA* gene expresses a 38kDa polypeptide which plays an important part in the process of recombinational DNA repair and homologous genetic recombination (Roca & Cox, 1990; Story *et al.*, 1992). It also is involved in the SOS response to DNA damage (Roca & Cox, 1990). Researchers, Clark & Margulies (1965) first described the *recA* protein during a study of recombination deficient *Escherichia coli* mutants.

The *recA* gene has been used for the study of bacterial evolution due its slow mutational rates over time (Eisen, 1995). The *recA* sequences from all genera of bacteria have used to produce separate corresponding branches on phylogenetic trees showing a high level of discrimination (Eisen, 1995). Due to its high resolving power the *recA* gene sequence has become widely used in bacterial evolution, taxonomic and phylogenetic studies.

The *recA* gene was found to be diploid with a single copy residing on each of the two large chromosomes present in the *B. cepacia* type strain 2993 (ATCC 25416). The two copies of the *recA* gene were found to be identical and provide a robust method for bacterial phylogenetic studies (Rodley *et al.*, 1995). Previous studies have found that there were nucleotide sequence polymorphisms within the *recA* gene and this was used to identify genomovars within *Burkholderia* (Mahenthiralingam *et al.*, 2000a; 2000b).

There are several species-specific *recA* gene primer combinations available that are used diagnostically (Mahenthiralingam *et al.*, 2000a, 2000b; Coenye *et al.*, 2001d; Vermis *et al.*, 2002). However, species-specific *recA* primers are yet to be developed for a vast number of species within the genus to allow allocation to genomovar. The lack of species-specific primers for some of *Burkholderia* species is due to the lack of heterogeneous sections within the gene sequence. For example, *B. cepacia* genomovar III originally contained two *recA* lineages, IIIA and IIIB. On further examination through DNA-DNA hybridisation studies it was revealed that another two *recA* lineages could be defined, these being genomovars IIIC and IIID (Vandamme *et al.*, 2003).

1.14 DNA-DNA Hybridisation

The quantitative measurement of whole genome DNA homology through renaturation rates has helped in determining the genetic relatedness amongst bacterial strains (De Ley *et al.*, 1970, Adnan *et al.,* 1993). This is achieved through the use of radio labelled and non-radio labelled DNA to anneal to fixed single stranded DNA (Forster *et al.*, 1985, Ezaki *et al.*, 1989). In past studies the DNA has been fixed onto nitrocellulose membranes to investigate the relative homology of the DNAs. DNA-DNA binding assays in micro-dilution wells using a fluorescent (non-radioactive) labelled DNA have in the past decade been an alternative to the isotope methods (Ezaki *et al.,* 1989, Balandreau *et al.,* 2001 Coenye *et al.*, 2001a, 2001b, 2001c, 2001d).

Through DNA-DNA hybridisation it is possible to assess the relatedness of the strains contained within a genus (Gillis *et al.*, 1995). From this, one may be able to further separate the strains within the genomovar should the values differ significantly, to create a more stringent method of taxonomic grouping (Vandamme *et al.*, 2003).

1.15 Aims and objectives

This study cover the collection of environmental samples and the isolation new environmental isolates of *Burkholderia* through the use of selective media *Pseudomonas cepacia* azelaic tryptamine (PCAT) agar plates and *Burkholderia* specific PCR analysis.

It will provide a detailed phenotypic characterisation of novel woodland *Burkholderia* isolates through colonial morphology, onion pathogenicity test, FAP, whole cell protein profiling and carbohydrate utilisation.

Novel woodland *Burkholderia* isolates are to be genetically characterised through BOX and ERIC PCR amplification and AFLP fingerprinting. DNA fingerprints will be compared with previously characterised isolates and integrated into pre-existing taxonomic groupings.

Investigate the possibility of niche-specificity, species-specificity and season-specificity within novel woodland *Burkholderia* isolates.

Screen all novel woodland *Burkholderia* isolates for the presence of the *cblA* gene. Any new *cblA* positive strains to be sequenced and compared with existing sequences of the *cblA* gene.

To amalgamate pre-existing 16S rDNA and *recA* gene sequence data with the novel *Burkholderia* isolates to create a phylogenetic tree. The phylogenetic tree will then be used to assess the relation of the novel isolates with fully characterised species within the genus.

To use the 16S rDNA gene sequence phylogenetic tree to investigate characteristics such as nitrogen- fixation and bioremediation of complex chemicals.

To design species-specific *recA* gene primers for novel woodland isolates and other *Burkholderia* species.

To investigate the DNA-DNA homology of the novel isolates to key type strains of *Burkholderia*. The DNA of the novel isolates will also be compared with each other to illustrate heterogeneity or homology within a woodland clade.

To investigate possible bioremedial properties in novel woodland *Burkholderia* isolates *via* solid and liquid culture enrichment studies. To identify the chemical by-products produced from bacterial bioremediation through gas chromatographic mass spectroscopy (GCMS).

1.16 Hypotheses for the study

Throughout the course of this thesis the following hypotheses will be upheld or rejected through experimental investigation.

1. There are common phenotypic characteristics present in *Burkholderia* isolates found in woodland habitats

2. Common genotypic characteristics are present in *Burkholderia* isolates found in woodland habitats

3. Novel woodland *Burkholderia* isolates can be clustered according to species of tree or plant

4. Environmental *Burkholderia* isolates have bioremedial properties.

5. Through polyphasic analysis, the clinical, plant pathogenic, environmental and bioremedial isolates can be clustered within their respective groups

6. To establish a new *Burkholderia* species through 16S DNA analysis of novel woodland isolates with other *Burkholderia* isolates and fully characterised species

CHAPTER 2

MATERIALS & METHODS

2.0 Background

During the course of this study, the several methods used will provide information on the phenotypic and genotypic profiles of the novel woodland isolates. This information will then be compared to known *Burkholderia* species.

2.1 Isolation of new environmental isolates of *Burkholderia* – Species-specificity

The ability of *Burkholderia* to establish itself within a variety of habitats has been noted through several studies (Kersters *et al.*, 1995, Achouak *et al.*, 1999). However, environmental isolates were the main focus of this study. These environmental *Burkholderia* isolates were obtained through the pseudo-random sampling of rhizospheric soil, leaf litter, plant roots and rotting wood. Details of the area, type of soil, plant or tree species were recorded for each of the samples, the information helped to trace back (to original sample) of any isolates found. The information was also used to investigate the species specificity potential of the novel *Burkholderia* isolates found within the environment. The samples of soil and plant material were obtained from woodlands sites in York, (United Kingdom), Kensington Gardens, London (United Kingdom) and Hampstead Heath, London (United Kingdom).

Sample material (0.5g fresh weight) was macerated in a grinding bag (Bioreba AG) containing 5ml of sterile distilled water (SDW). Appropriate dilutions of the bacterial suspensions were made (ranging from 10^{-1} to 10^{-4}) using SDW and 200µl of each dilution was spread out onto *Pseudomonas cepacia* azelaic tryptamine (PCAT) agar plates (see appendix A1). The dried inoculated PCAT plates were incubated aerobically at 28°C for 3-5 days. Bacterial colonies were subsequently selected and sub-cultured onto nutrient agar for further tests on proviso that they were displaying *Burkholderia*-like characteristics and were not in contact with fungal growth. Where possible a maximum of 5 colonies were selected from each sample (see appendix A3 for sub-culturing streaking method for bacterial cultures). Each *Burkholderia*-like culture was nominated a name based on area isolated and type of sample (see appendix A7 for description). Stock cultures were suspended glycerol broth and stored

at -80°C (see appendix A1 for glycerol broth). Working cultures were sub-cultured on to nutrient agar plated every 3 days to ensure viable cultures for use. After 2 weeks cultures were discarded to ensure the genetic changes are not introduced due to constant sub-culturing and new working cultures were used.

2.1.1 Gram stain.

The bacterial isolates recovered from the samples were observed under a microscope to define the cellular morphology and to confirm the presence of Gram negative rods. (one of the physical characteristics of *Burkholderia*). A clean microscope slide was passed through a Bunsen flame and a bacterial colony was aseptically transferred onto a drop of water on the microscope slide and emulsified to form a slightly turbid suspension. The bacterial suspension was dried at room temperature (RT°C). Once dried the bacterial cells were fixed onto the slide by passage through a Bunsen flame.

The slide was then flooded with 2% w/v crystal violet solution (see appendix A3) and left for 1min. The slide was rinsed with demineralised water then flooded with Grams iodine solution (see appendix A3) and left for 1min, after which it was rinsed with demineralised water. The bacterial cells were then counterstained with safranin (see appendix A3) for 15s, the slide was rinsed with demineralised water, dried gently by blotting on absorbent paper before being examined microscopically.

2.1.2 Cytochrome oxidase test.

This test identifies the enzyme cytochrome *c* oxidase, which is found in obligate aerobic bacteria. A piece of filter paper was soaked in a fresh colourless solution of 1% (w/v) N-N-N'-N'-tetramethyl-p-phenylenediamine dihydrochloride in a Petri dish. A small amount of a 24h agar plate culture was transferred onto the filter paper, a purple-blue colour observed within 10s indicates a positive result.

2.2 *Burkholderia* - specific polymerase chain reaction (PCR) amplification

This particular PCR analysis amplifies a region of the 16S rDNA gene specific to *Burkholderia* species (O'Callaghan *et al.*, 1994). The use of this PCR, generating a 770bp sized amplicon, indicates the presence of *Burkholderia* DNA within the sample. This PCR amplification was one of the tests used to assist the characterisation process of *Burkholderia* isolates.

2.2.1 Total DNA extracts by alkali lysis.

Total DNA extracts (lysates) of the presumptive *Burkholderia* isolates were prepared by emulsifying 1 - 2 colonies of an overnight agar plate culture into 100µl of 50mM NaOH solution in an microcentrifuge tube. The bacterial suspension was boiled for 7min at 100°C to lyse the cells, then cooled rapidly on ice and diluted by adding 900µl of SDW.

2.2.2 *Burkholderia* - specific PCR amplification.

For each PCR amplification a 25µl reaction was used. This comprised of DNA (2.5µl); primers PC480 and PC1240 (1µM each) (Genosys) (see appendix A10 for primer sequences); deoxynucleotide triphosphates (dNTPs) (200µM each) (Boeringer-Mannheim); *Taq* polymerase (1.25 Units) (Promega); $MgCl_2$ (2.5mM) (Promega) and 1x buffer (containing Tris-HCl, $MgCl_2$ and Triton X-100) (Promega). Denaturation of the template DNA was achieved through heating at 95°C for 5 min. Amplification was for 25 cycles each consisting of 15s at 96°C; 30s at 54°C and 30s at 70°C. Following the amplification cycles the samples were then incubated at 70°C for 10min.

2.2.3 Electrophoresis of PCR products.

The PCR products generated were separated by electrophoresis in a 1% agarose gel made with 1 x TAE buffer (see appendix A2) at 80V for 40-60 min in a gel tank containing 1x TAE buffer. A 100bp ladder (Marker XVI) (Boehringer-Mannheim) was used to measure the size of the fragment generated.

The separated PCR product(s) within the gel was then stained with ethidium bromide (final concentration within solution was 100µg /ml) for 30 min and then the gel was destained with demineralised water for 1 min. The image of the DNA within the gel was obtained through the Multianalyst imager (BioRad).

2.3 Phenotypic characterisation of novel *Burkholderia* isolates

Once a tentative genus allocation was given to the novel *Burkholderia* isolates defining the phenotypic characteristics was initiated. This involved identifying various phenotypic aspects such as colonial morphology, fatty acids, carbohydrate utilization and protein profiles.

Polyphasic taxonomy and how it could be used to create comprehensive taxonomic grouping of members within a genus will be addressed in later chapters. However, it is necessary to collect both physical and genetic profiles of the novel *Burkholderia* isolates collected in this study before further analysis. It may be possible to link the physical characteristics (phenotype) with particular genetic characteristics (genotype). Through studying novel *Burkholderia* isolates, it is hoped that certain environmental conditions (such as type of tree species, soil, exposure to chemical) may affect the genotype of the isolate, giving rise to common characteristics found within a group or cluster.

2.4 Fatty acid profiling

Fatty acid profiling was implemented as a method used to confirm the *Burkholderia* genus allocation provided by the *Burkholderia* specific PCR analysis. Each genus of bacteria has a distinctive fatty acid / fatty acid methyl ester (FAME) profile (Stead, 1988; Sasser, 1990). From the use of this method one can confirm that the isolate was indeed a *Burkholderia*. Bacterial isolates for fatty acid profiling (FAP) were inoculated (by a streaking method, see appendix A6) onto 2 plates of fresh trypticase soya broth agar (TSBA) (see appendix A1) and incubated aerobically at 28° ± 1°C for 24h ± 1h.

The cells were harvested from the third quadrant (containing cells in the stage of exponential growth) from each plate by using a plastic loop and deposited on the bottom of a clean dry test tube. The amount required for FAP was approximately 40mg. The cells were harvested and saponified with 1ml of reagent 1 (see appendix A6), vortexed and placed in a boiling water bath for 30min. The samples were methylated by adding 2ml of reagent 2 (see appendix A6) vortexed and placed in an 80°C water bath for 10min. Extraction of the FAMEs was achieved through the addition of 1.25ml of reagent 3 (see appendix A6); the tubes were rotated for 10min on a rotary mixer, the bottom solvent phase was then removed. Finally, a base wash was carried out through the addition 3ml of reagent 4 (see appendix A6) to each tube and mixed on the rotary mixer for 5min.

The addition of several drops of saturated NaCl solution made the two phases apparent and the top solvent phase was removed for gas chromatographic analysis

(Hewlett Packard GC model 5890). Of each sample, 1µl was injected (injector temperature 250°C) into a fused silica capillary column (column temperature 270°C) with hydrogen as the carrier gas. The retention time of the samples in the column was 20min. The peaks were integrated and named through the use of a peak-naming component in the HP 5898A Microbial Identification System (Newark, Delaware, USA). Through the use of this identification system, bacterial genera and species were identified. The presence of hydroxy acids is of great importance in the identification of Gram negative bacteria (Stead, 1992). The species of *Burkholderia* are specified through the ratios of the hydroxy acids and other selected fatty acids (Stead, 1992). The fatty acids present in plant-associated bacteria are typically between 9 and 20 carbon atoms in length (Sasser, 1990). The presence of these specially sized fatty acids within bacteria can identify them easily as possible plant pathogens.

2.5 Growth of environmental *Burkholderia* isolates at 28°C and 37°C

The ability of the environmental isolates to grow at 28°C and 37°C may indicate the potential of an environmental isolate to colonise humans and to possibly cause infections. The novel isolates were cultured onto nutrient agar plates and incubated at 28°C and 37°C for 24-48h aerobically. After incubation signs and levels of growth were determined and compared visually.

2.6 Onion pathogenicity test

The onion pathogenicity experiment determined the ability of the *Burkholderia* isolate to infect and to develop the characteristic sour rot in onion tissue. The presence of yellowing and slimy nature of the tissue and the distinctive sour smell indicative the pathogenic nature of the isolate to onions.

The *Burkholderia* isolates were subcultured onto nutrient agar plates and incubated for 48h at 28°C. The bacterial cells were then resuspended into 5ml PBS and adjusted to OD 0.1 at 600nm. The outer scales of the onion were carefully removed and squares of 3cm^2 were cut from the scales with a sterile scalpel blade. The scales were then placed into a sterile Petri dish containing filter paper soaked with SDW. The centre of

the onion scale was pierced with a sterile syringe needle and 25µl of the bacterial suspension was applied over the lesion.

Inoculated onion scales were incubated aerobically for 72h at 28°C. Sterile distilled water and a non-onion pathogenic strain of *B. cepacia* were used as negative controls. After the incubation period the onion scales were examined visually for signs of maceration and whether they were emitting the characteristic 'sour rot' smell.

2.7 Carbohydrate utilisation of *Burkholderia* species.

Different bacteria vary in their ability to utilise carbohydrates, through this characteristic, a profile of the isolate can be formed (Burkholder, 1950, Richard *et al.*, 1981 and Gillis *et al.*, 1995). Which may go on to define the isolate to genus level. Through the profile of carbohydrate utilisation it is possible to determine the genus or even the species of bacteria after the initial Gram stain analysis.

The API identification kits (Biomérieux) contain a range of different carbohydrate tests and these were used to create a carbohydrate utilisation profile of novel isolates of *Burkholderia*. The identification strips (API 20NE and API 50CH) were used according to the manufacturer's instructions. The results were read after a 24h and 48h incubation period at 28°C. The positive and negative growth results were recorded to produce a numerical code, when this code was referred to the manufacturer's database provided a bacterial identification of the isolate.

2.8 Whole cell protein profiling of *Burkholderia* species- SDS PAGE

The extraction of proteins from the *Burkholderia* isolates was based on a method first devised by Laemmli (1970). Through the extraction of the proteins, it is hoped a protein profile specific to novel woodland *Burkholderia* isolates could be identified. It was also hoped that species specificity of the *Burkholderia* isolates could be highlighted in differences in the protein profile (presence or absence of protein bands).

2.8.1 Protein extraction from Gram negative bacteria.

The bacteria cultures were grown for 48h at 28°C on glucose yeast extract chalk agar (GYCA) plates (see appendix A1). The bacteria were then suspended in 5ml of NaPBS buffer (see appendix A4) and mixed thoroughly *via* vortexing. The bacterial suspension was then used to inoculate 3-4 GYCA plates which were then incubated for 48h at 28°C. After incubation each bacterial culture was washed and resuspended with NaPBS to a final volume of 30ml of bacterial suspension which was centrifuged at 10,000 rpm for 10min at 4°C. The bacterial cultures were washed with NaPBS and centrifuged a further two times. A minimum of 50mg of bacterial cells was required for protein extraction. The optimum weight of 100mg of bacteria was treated with 1.8ml of sample treatment buffer (see appendix A4), thoroughly mixed via vortexing.

The resuspended bacteria were lysed with SDS solution (see appendix A4) to a final concentration of 2%, mixed thoroughly *via* vortexing then boiled at 95°C for 10min. The lysed cells were then rapidly cooled on ice for 5min and centrifuged at 14,000rpm for 3min. The supernatant was transferred to a clean microcentrifuge tube for storage at -80°C or for electrophoresis.

2.8.2 Separation of proteins *via* SDS PAGE electrophoresis.

The supernatant containing the protein extract was subsequently separated at 2°C on a 5% stacking gel and 12% separation gel (see appendix A1 for preparation of SDS polyacrylamide gel) by electrophoresis for 3.5h at 180V.

The polyacrylamide gel was stained using a Coomassie blue staining solution (see appendix A4) for 45min, then decolourised using a destaining solution (see appendix A4) overnight. The gel was visualised using a densiometer imager (BioRad).

2.9 Genotypic characterisation of novel *Burkholderia* isolates

To supplement the data gained through the biochemical and physical properties of novel *Burkholderia* isolates, several molecular methods have been utilised or devised. These various methods were used to generate a range of genetic profiles which were used individually or combined to observe clustering of novel *Burkholderia* isolates and fully characterised *Burkholderia* species. These molecular methods used either random polymorphic regions across the whole bacterial genome or highly conserved

gene regions within the *Burkholderia* genome. The collation of genotypic data would enable any common genetic characteristics between the various groups of *Burkholderia* to be highlighted in this study.

2.10 BOX element (BOX) and enterobacterial repetitive intergenic consensus (ERIC) PCR analysis.

BOX element PCR is a technique which amplifies a repetitive sequence along the genomes (Versalovic *et al.*, 1994). This method was chosen as a taxonomic tool as it was used a previous study by Jane Richardson as part of her PhD studies. It was hoped that the BOX PCR data from this study could be combined with the work previously carried out to expand the range of isolates used to highlight any species-specific groups. It was also hoped that the robustness of this method could be tested between two operators at difference time points.

2.10.1 Isolation of genomic DNA from *Burkholderia* species.

Three small colonies from an overnight agar culture were emulsified with SDW in a 1.5ml microcentrifuge tube and centrifuged to pellet the cells. The supernatant was removed and the cells resuspended in 480µl of SDW. The DNA was extracted using the DNA Wizard Kit (Promega) according to the manufacturer's instructions.

2.10.2 BOX PCR amplification.

The PCR amplifications were carried out in 25µl reactions containing template DNA (50ng); primer BOXA1R (50pM) (see appendix A10 for primer sequence); dNTPs (200µM) (Amersham- Pharmacia), 1x PCR buffer (containing 15mM $MgCl_2$) (Perkin-Elmer) and Ampli*Taq* DNA polymerase (2 units) (Perkin-Elmer). Denaturation of sample DNA was at 95°C for 7 min. Amplification was for 30 cycles of 1min at 94°C, 1 min at 40°C and 8min 65°C, samples were then incubated at 65°C for 16min.

2.10.3 ERIC PCR amplification.

The PCR amplifications were carried out in 25µl reactions containing template DNA (50ng); primers ERIC1-R and ERIC2 (50pM each) (see appendix A10 for primer sequences); dNTPs (200µM), 1x PCR buffer (containing 15mM $MgCl_2$) and Ampli*Taq* DNA polymerase (2 units) (Perkin-Elmer). Denaturation of sample DNA was at 95°C

for 7min. Amplification was for 30 cycles of 1min at 94°C, 1min at 52°C and 8min at 65°C; samples were then incubated for 16min at 65°C.

2.10.4 Electrophoresis of fingerprinting PCR products (BOX and ERIC).

The PCR products were separated by electrophoresis in a 2% agarose-TAE gel at 90V for 2-3h in a tank containing 1X TAE buffer. The gel was stained with ethidium bromide (final concentration of 100μg / ml) for 30-45min on an orbital shaker and destained with demineralised water for 1min before visualising under UV at 355nm.

The DNA fingerprints were then analysed through the GelCompar version 4.0 (Applied Mathematics) program to align bands and to observe any similarities in the DNA profiles generated through these PCR techniques.

2.11 Phylogenetic comparison of gel patterns by GelCompar computer analysis

The electrophoresis patterns for the BOX, ERIC and AFLP reactions were analysed with GelCompar version 4.0 software. The use of this software enabled the fingerprints generated to be aligned (through expansion or compression of the gel image when aligned with DNA markers on the gel image) for analysis.

For conversion of gels the settings were as follows, resolution was set at 555, rescaling was applied to each track, curve smoothing was 0, 3 nodes were used, spline thickness was 4 and gel strip thickness was 12. The peaks were then aligned. The reference lane for normalisation was used for Marker XVI on each of the gels and rescaling of the DNA fingerprints was applied. The final dendrograms were generated using unweighted pair-grouping (UPGMA) algorithm with fine alignment for cluster correlation.

Table 2.1 Description of *Burkholderia* species from various culture collections used in the study

The isolates described in the table below are either fully characterised species of *Burkholderia* from established culture collections or partially characterised isolates of *Burkholderia* from previous research projects. The colonial morphologies described below were obtained from information forms that were provided with the culture or from records in culture collections.

ID Number	Organism	Isolation Media*	Colonial Morphology**	Origin
NCPPB 2993	*B. cepacia* (Type Strain)	NA	Bright yellow pigment, small domed.	
P410 / RR8	*B. gladioli pv agaricola*			Mushroom isolate.
NCPPB 1051	*B. gladioli pv. agaricola*		Dry, small cream and wrinkled.	Gladioli isolate. Zimbabwe.
P3207a	*B. gladioli*			
P165	*B. gladioli pv allicola*			
NCPPB 1970	*B. gladioli pv. allicola*			
J2315	*B. cepacia*		Small, matt colonies, brown diffused pigment.	Clinical. CF patients. Edinburgh- Toronto epidemic lineage.
P3405 / SH1	*B. gladioli*	TB-T	Wrinkled, small, dry, slight yellow pigmentation.	Environmental. Leek Field. Shifnal, Shropshire, UK.
P3361 / C1576	*B. cepacia*			Clinical. CF patients. UK. J.W.R Govan.
P3345 / RW36	*B. cepacia*	PCAT	Yellow/ green pigmentation. Domed and shiny.	Environmental. Rotting wood. Sand Hutton, York, UK.
LMG 2161	*B. cepacia*		Shiny, domed. Green iridescent pigmentation, grape like smell.	Environmental, soil, Trinidad.
LMG 10929	*B. vietnamiensis*			Environmental. Soil.
LMG 13010	*B. multivorans*		Shiny, domed shaped. Slight green pigmentation.	Clinical, CF, Belgium.
LMG 14191	*B. pyrrocinia*			Soil
LMG 16225	*B. fungorum* (type strain)			Fungus, *Phanerochaete chrysosporium*, Grenoble, France.
LMG 16232	*B. vietnamiensis*		Small, cream, shiny. 'Rice-like' smell.	Clinical, CF, Sweden.
LMG 16307	*B. fungorum*			Clinical, cerebrospinal fluid.
LMG 16654	*B. cepacia*			Clinical, CF, UK.
LMG 16656	*B. cepacia*		Small, cream, shiny domed. Diffused brown pigmentation.	Clinical, CF, Edinburgh.
LMG 16659	*B. cepacia*		Small, domed shaped, matt.	Clinical, CF, Edinburgh.
LMG 16660	*B. multivorans*			Clinical, CF, Glasgow.
LMG 16665	*B. multivorans*		Small, domed, green, iridescent pigment, 'grape-like' smell.	Clinical, non-CF brain abscess, UK.
LMG 17997	*B. cepacia*			Clinical, non-CF urine, Sweden.
LMG	*B. cepacia*		Small, domed shaped with	Clinical, CF, Canada.

ID Number	Organism	Isolation Media*	Colonial Morphology**	Origin
18821			diffused brown pigment.	
LMG 18836	*B. vietnamiensis*		Small, cream, shiny domed. 'Rice-like' smell.	Clinical, chronic granulamatousis. Canada.
LMG 18863	*B. cepacia*		Small, cream / pearly, matt.	Clinical, CF, Canada.
LMG 18870	*B. stabilis*		Small, cream / pearly coloured, shiny domed.	Clinical, CF, Canada.
LMG 18888	*B. stabilis*		Small, cream coloured, shiny. 'Rice-like' smell.	Clinical, blood, Belgium.
LMG 19076	*B. caledonica* (type strain)			Soil rhizosphere, Edinburgh, UK.
LMG 19182	*B. ambifaria* (type strain)		Two colony types: small and medium cream colonies.	Pea rhizosphere, Wisconsin, USA.
LMG 19450	*B. sacchari* (type strain)			Sugar cane plantation soil, Brazil.
LMG 20227	*B. fungorum*			*Phanerochaete chrysosporium.* Fungus, Grenoble, France
LMG 20594	*B. terricola*			Agricultural soil
LMG 20598	*B. hospita*			Agricultural soil.
LMG 20983	*B. anthina*			Patient suffering from CF, Sputum, Blackpool, UK.
P3588 / 97-37A	*B. cepacia*			*Allium cepa* - Onion.
P3589 / 99-3	*B. cepacia*			*Allium cepa* - Onion.
P3590 / 99-10	*B. cepacia*		Small cream with diffused yellow pigment.	*Allium cepa* - Onion.
P3591 / 63-75	*B. cepacia*			*Allium cepa* - Onion.
P3592 / 99-7	*B. cepacia*		Small, shiny and domed shaped colonies. Yellow pigment.	*Allium cepa* - Onion.
P3593 / 99-11	*B. cepacia*		Matt, pearly, small domed shaped. Yellow pigment.	*Allium cepa* - Onion.
P3594	*B. cepacia*		Small, shiny, domed. Yellow pigment.	*Allium cepa* - Onion.
P3595 / 98-1	*B. cepacia*		Small, shiny and domed. Yellow pigment.	*Allium cepa* - Onion.

*** - No description available unless indicated otherwise.**

**** - unless indicated otherwise, colonial morphology is small, cream and dome shaped.**

P- Protect bacterial culture library at the Central Sciences Laboratory (DEFRA), Sand Hutton, York.

NCPPB- National Collection of Plant Pathogenic Bacteria (UK).

LMG- Laboritorium voor Microbiologie, Universiteit Gent (Belgium).

2.12 Amplified fragment length polymorphism (AFLP) analysis (Vos *et al.*, 1995)

Through the use of this method, it was hoped that grouping of the novel *Burkholderia* isolates and *Burkholderia* species would be made possible by creating a highly discriminative whole genome DNA profile.

2.12.1 Modification of template DNA by double restriction digests.

Template DNA was prepared as described in section 2.3 (Promega Wizard Genomic DNA Purification Kit); a double digest was carried out through the use of *Eco*RI and *Mse*I (Promega) restriction endonucleases.

A 20µl digest of template DNA was incubated at 37°C for 1h with *Eco*RI (5 units) in 1x buffer H (Promega) and bovine serum albumen (BSA) (50ng / µl) (Promega). A second digest with *Mse*I was carried out at 65°C for 2h through the addition of *Mse*I (5 units) (Promega) in 1x buffer F.

The preparation of the double digested DNA was carried out using reagents from the Microbial fingerprinting kit (Perkin-Elmer, Applied Biosystems). Ligation of double stranded adaptor / linker molecules was achieved by the addition of *Eco*RI (2µM) adaptor, *MSe*I (20µM) adaptor, T4 DNA ligase (0.33 unit), ligation buffer (1x) and 5µl digested DNA. The reaction was left to ligate overnight at room temperature.

2.12.2 Preselective PCR amplification of fragments with *Eco*RI and *Mse*I adaptors.

The preselective PCR amplification was carried out in a 20µl reaction containing PCR buffer with 15mM $MgCl_2$ (1x), 25pm dNTPs, 25pm primer MOO (Perkin-Elmer, Applied Biosystems), 25pm primer EOO (Perkin- Elmer) 0.4units of *Taq* polymerase and 5µl ligated DNA. The DNA was denatured at 94°C for 2 min. Amplification was for 20 cycles at 20s at 94°C, 30s at 56°C and 2min at 72°C. After amplification the samples were left to incubate for 30min at 60°C. The products from the preselective PCR were separated on a 2% TAE electrophoresis gel at 80 V for 1h. The products from the preselective PCR were then diluted twenty-fold and used for selective PCR amplification.

2.12.3 Selective PCR amplification of *Eco*RI and *Mse*I-modified fragments.

The selective PCR amplification was carried out in a 10µl reaction containing AFLP-*Mse*I primer (5µM) (Perkin-Elmer, Applied Biosystems), AFLP-*Eco*RI-C primer with NED fluorescent label (1µM) (Perkin-Elmer, Applied Biosystems), AFLP core amplification mix (contains *Taq* polymerase) (Perkin-Elmer) and 1.5µl diluted preselective PCR product. The DNA was denatured for 2min at 94°C. Amplification was for 10 cycles consisting of 20s at 94°C, 30s at 66°C* and 2min at 72°C. The PCR

products were then left to incubate at 60° for 30min. After incubation the PCR products were separated by acrylamide gel electrophoresis.

*Annealing temperature drops 1°C after each cycle.

2.12.4 Acrylamide gel electrophoresis of AFLP amplification products.

The selective PCR products were diluted to a ratio of 1:2 with loading buffer mix containing fluorescent ROX-500 size standard (see appendix A2 for components) (Perkin-Elmer, Applied Biosystems), denatured at 95°C for 5min before loading onto a 5% acrylamide sequencing gel (see appendix A1 for Sequagel^{TM} sequencing gel composition). The selective PCR products were separated by electrophoresis (well to read distance of 36cm) on a polyacrylamide gel for 4 hours at 2500 volts on a Perkin-Elmer ABI Prism 377 sequencer. The parameters of the sequencing gel electrophoresis were set according to the manufacturers instructions (Perkin-Elmer, Applied Biosystems).

The GeneScan analysis program affiliated with the sequencer enabled the gel lanes to be tracked and its trace to be extracted. The extracted lanes were then viewed as densitometric traces to assess how sharp the peaks were and to subtract the 'background noise' generated during the scanning process.

The AFLP banding patterns were then converted, aligned and analysed through the use of the ABICONV program (Applied Mathematics). The banding patterns were aligned through normalisation against a reference marker for the ROX size standard. Each lane contained ROX size standard and subsequently the gel track were normalised (aligned) against each other. The phylogenetic relatedness of the *Burkholderia* isolates was performed using an unweighted pair group mean average (UPGMA) analysis.

2.13 Sequencing of the 16S rDNA gene

The comparison of a gene sequence which mutates little over a long period of time (or a highly conserved gene) such as the 16S rDNA gene, can reveal the relationships between two or more isolates. The more mutations found between gene sequences indicates a decrease in relatedness of the isolates in question. Through comparing the

16S rDNA sequences of novel *Burkholderia* isolates and *Burkholderia* species, a phylogram be created and specific clusters identified.

The genomic DNA was prepared using the Promega Wizard Genomic DNA purification kit according to the manufacturer's instructions.

The PCR amplifications were carried out in 50µl reactions containing 50ng of DNA; primers 27F and PC1240 (1pm) (Sigma-Genosys) (see appendix A10 for primer sequences), dNTPs (8mM) (Promega), *Taq* polymerase (0.5U) (Promega, UK) $MgCl_2$ (12.5mM) (Promega) and 1X buffer (Promega). Denaturation of the DNA was at 95°C for 3min. Amplification was for 35 cycles consisting of 1min at 94°C, 1min at 55°C and 1min at 72°C; the samples were then left to incubate for 4min at 72°C.

2.13.1 Purification of PCR products with PEG solutions.

The PCR products were purified through the use of a PEG solution (see appendix A5). The PEG solution selectively precipitates DNA of more than 150bp in size to leave primers, nucleotides and truncated PCR products in the supernatant (Rosenthal *et al.*, 1993). Equal amounts of the PEG solution were added to each PCR reaction mixed thoroughly by vortexing and left to incubate at room temperature (RT°C) for 30min. The mixture was centrifuged for 15min at 14,000 rpm and the supernatant was removed before the addition of 500µl ethanol at -20°C. The DNA was allowed to precipitate overnight for a high yield at -20°C, the mixture was then centrifuged at 14,000 rpm for 30min.

The supernatant was removed and the DNA pellet washed with 500µl of 70% ethanol at -4°C, vortexed and centrifuged for 15min at 14,000 rpm. The supernatant was removed and the DNA pellet was left to air dry at RT°C and resuspended in 10µl sterile distilled water (SDW). The purified PCR product was sequenced with the primers used in the PCR analysis and an internal primer PC480 (see appendix A10 for primer sequence).

The sequences were then analysed through the use of the GCG program (Devereaux *et al.*, 1984). The sequences were then aligned with the 16S rRNA sequences from other *Burkholderia* isolates through the use of the NCBI-ClustalX program (Thompson *et al.*, 1997) and from this, a bootstrapped phylogram was created. The sequences of other *Burkholderia* 16S rRNA genes were obtained from the National Centre for

Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). From the phylogram created, it was possible to determine how the novel environmental isolates related to fully characterised species within the *Burkholderia* genus.

2.14 Restriction fragment length polymorphism (RFLP) patterns of 16S rDNA PCR products

The use of RFLPs in a highly conserved region like the 16S rDNA gene will generate a small range of restriction patterns for comparison. Through this method, it is hoped that clustering of novel *Burkholderia* isolates could be made possible.

The 1.2kb DNA fragment generated from the 16S rDNA gene PCR amplification was used to generate a RFLP pattern to determine any differences between the novel isolates. The 16S rDNA gene fragments were cut using a restriction enzyme *Dde*I. Restriction digestions were carried out in 14μl reactions at 37°C for 3h containing 16S rDNA PCR product (1μg), restriction endonuclease *Dde*I (0.8 unit) and buffer C (1x). Digested PCR products were separated by electrophoresis in a 1.5% TAE agarose gel containing 100μg ethidium bromide before visualisation under UV light at 355nm.

2.15 *RecA* gene PCR analysis

Burkholderia cepacia has been described as a 'complex' containing nine genomic species or genomovars to date (Coeyne & LiPuma, 2002). It seems that the nine genomovars are capable of causing infections in immuncompromised humans. In recent studies it has been shown that *B. cepacia* genomovar III can be frequently found within the environment such as in certain soil types and the rhizosphere of certain plants such as maize (Balandreau *et al.*, 2001 and Fiore *et al.*, 2001).

The determination of genomovar status has been made possible through the use of *recA* gene sequencing (Mahenthiralingam *et al.*, 2000a, 2000b). Some primer designs have been used to be species-specific.

A range of primers were designed based on the *recA* gene data from various members *β-Proteobacteriacae* group (*Ralstonia eutrophia*, *Bordetella pertussis*,

Pseudomonas aeruginosa, *Xanthomonas campestris*, and *Methylobacillus flagellatum*) and *B. cepacia.* This enabled a set of primers that would amplify along sections of the *recA* gene.

Table 2.2 *β-Proteobacteriacae recA* gene sequence accession numbers used in *recA* analysis

The following *β-Proteobacteriacae recA* gene sequences were obtained from the GeneBank sequence database. Through aligning these *recA* gene sequences, generic primers could be designed to allow the amplification of any potential *recA* gene sequences from the novel *Burkholderia* isolates. Through developing a specific *recA* gene primer a genomovar (species) status could potentially be allocated to novel *Burkholderia* isolate or *Burkholderia* species not yet contained with any of the existing genomovars.

Taxon and Name	GeneBank accession number
Ralstonia eutropha	AF312928
Xanthomonas campestris	AF399933
Bordetella pertussis	X53457
Methylobacillus flagellatum	M35325
Pseudomonas aeruginosa	X05691

2.15.1 *RecA* gene PCR amplification.

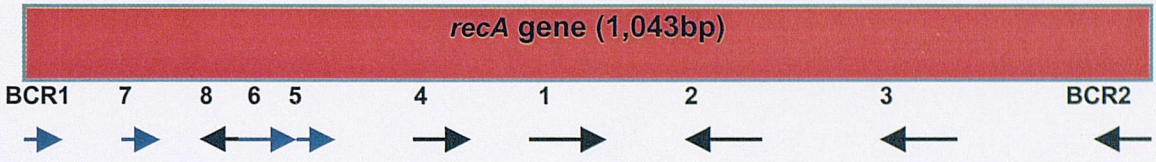
The total genomic DNA was prepared through NaOH lysis as previously described. The PCR amplifications were carried out in 50μl reactions containing template DNA (100ng), primers BCR1 and BCR2 (Mahenthiralingam *et al.*, 2000a), RecA1, 2, 3, 4, 5, 6, 7 and 8 (see appendix A10 for primer sequences and positions) (2.5pm), dNTPs (2mM) (Boehringer-Mannheim), 1x Red*Taq* buffer and Red*Taq* polymerase, 1.25 units (Sigma). Amplification was for 30 cycles consisting of 1min at 94°C, 1min at *44-62°C and 2min at 72°C, the samples were then left to incubate for 10min at 72°C.

* Dependent on primer combination used for amplification.

The PCR products (25μl) generated were then run on a 1% TAE agarose gel for 45mins photographed and measured. The DNA from the agarose gel was then

subsequently transferred onto nitrocellulose membrane *via* the Southern blotting method. The blot was then probed with a P_{32} labelled fragment of the *recA* gene generated from strain J2315. The membrane was then visualised using photographic film so that areas of hybridisation could be determined.

Figure 2.1: Direction of primers used in *recA* gene sequencing of *Burkholderia* isolates



2.16 Species - specific *recA* PCR analysis for *B. fungorum* and *B. sacchari*

Burkholderia fungorum and *B. sacchari* were not allocated within predefined genomovars at the time of this study. Through amplifying *recA* sequence data of these two species, it was hoped that a species-specific primer set could be designed. This in turn could result in a formation of a new genomovar, containing *Burkholderia* isolates characteristic of either of these species.

From the data generated from the *recA* gene PCR using a range of primers it was possible to design a set of primers for *B. fungorum* and *B. sacchari* which were species specific. These diagnostic PCR primers enabled the generation of amplicons sized 330bp and 248bp respectively.

The total genomic DNA was prepared through NaOH lysis as previously described in section 2.2.1. The PCR reactions were carried out in 25µl reactions containing template DNA (100ng), primers FunF, FunR (*B. fungorum*), SacF and SacR (*B. sacchari*) (see appendix A10 for primer sequences) (2.5pm), dNTPs (2mM) (Boehringer-Mannheim), 1x Red*Taq* buffer (containing $MgCl_2$) and Red*Taq* polymerase (1.25units) (Sigma). Denaturation of the DNA was at 94°C for 5mins. Amplification was for 30 cycles consisting of 1min at 94°C, 1min at 67°C and 2min at 72°C, the samples were left to incubate for 10min at 72°C.

The PCR products are electrophoretically separated on a 1% TAE agarose gel for 45min at 80V before examination under UV light.

2.17 Transfer of DNA onto nitrocellulose membrane *via* Southern blotting

DNA fragments were separated by electrophoresis on an agarose gel as described previously. The gel was rinsed with distilled water and soaked in 0.25N HCl for 25min to acid nick the larger fragments of DNA. The Southern blot apparatus was set up with 0.4M NaOH as the transfer solution (Sambrook *et al.,* 1989). DNA transfer was left over night. After the blotting process the Hybond-N+ (Amersham-Pharmacia) was rinsed briefly with 2X SSPE (see appendix A2) before the DNA was bound onto the membrane using a UV Cross-linker Stratalinker® 1800 (Stratagene).

2.18 DNA-DNA hybridisation of the P^{32} labelled *recA* gene of J2315 to *recA* PCR amplicons from novel *Burkholderia* isolates.

The hybridisation of single strand DNA is only possible when the two strands of DNA have some degree of homology. The more homology present between the two strands the stronger the DNA strands will anneal to each other.

2.18.1 Pre-hybridisation of the DNA blot.

The nitrocellulose (Hybond- N+) membrane was prehybridised with 25ml of a solution containing SSPE (5X), Denhardt's solution (5X) (see appendix A5), sheared denatured salmon sperm DNA (20μg/ ml), 0.5% SDS and SDW. Pre-hybridisation was achieved by placing the blots in a soda glass tube and incubated at 65°C for 2h in a rotating hybridisation oven.

2.18.2 Preparation of the oligoprobe and hybridisation process.

Oligolabelling reactions were performed by the methods of Feinberg & Vogelstein (1983). This method produced a probe which was both specific and gave a good radioactive signal. The *recA* gene fragment (50ng) to be used for a probe was boiled for 5min before the addition of the other components. The *recA* DNA probe was in a 50μl reaction containing 1X labelling buffer (Promega), dGTP (0.8mM), dATP (0.8mM), dTTP (0.8mM) (Boehringer Mannheim), 1.5 units of αP^{32} dCTP (50μCi:3000

Ci/mmol) (Amersham-Pharmacia Biotech), nuclease-free BSA (20μg) (Promega) and 1 unit Klenow fragment of DNA Polymerase (Promega). The *recA* probe was then incubated at RT°C for 60min.

The *recA* probe was then denatured by heating at 95-100°C for 5min and rapidly chilled on ice before adding to the prehybridisation solution. The *recA* probe was left to hybridise with the bound DNA overnight at 65°C in a hybridisation oven. The hybridisation solution was then removed and the nitrocellulose membrane was washed with a series of stringency solutions containing SSPE and SDS (see appendix A2 for wash solutions). The washed membrane was then blotted dry and covered in saran wrap and subsequently auto-radiographed in a cassette with medical X-ray film (Fujifilm) for 45min at -70°C for higher intensity.

2.19 Potential nitrogen fixation in novel isolates *nif*K PCR Amplification.

This PCR analysis was designed to generate a 1,080 bp fragment of the *nif*K gene which codes for the β-subunit of the nitrogenase MoFe protein. The reactions were carried out in 50μl reactions containing 100ng of template DNA primers *nif*KF and *nif*KR, (2.5pm) (Minerdi *et al.*, 2001) (see appendix A10 for primer sequences), dNTPs (2mM) (Boehringer-Mannheim), 1x Red*Taq* buffer and Red*Taq* polymerase (1.25 units) (Sigma Aldrich). Denaturation of the DNA was for 5min at 95°C. Amplification was for 30 cycles consisting of 1.5min at 95°C, 1min at 62°C and 2min at 72°C, the samples were then let to incubate for 10min at 72°C.

Positive controls used in this PCR amplification were *B. vietnamiensis* LMG 10929 and LMG 16232. PCR products were separated on a 1x TAE agarose gel through electrophoresis and visualised under UV light at 355nm.

2.20 Agarose gel extractions

After gel electrophoresis, DNA fragments of interest were excised from the agarose gel and purified using the Qiaex II gel extraction kit (Qiagen) according to manufacturer's instructions. The extracted and purified DNA fragment was eluted into 20μl of sterile distilled water. The purified DNA could then be used for direct

sequencing or inserted into a cloning vector to further increase the number of DNA strands for other analyses.

2.21 Preparation of competent JM109 cells using $CaCl_2$ treatment (Sambrook *et al.*, 1989)

Escherichia coli strain JM109 was used to make competent cells for heat shock transformation. *E. coli* strain JM109 has the genotype of *end*AI, *rec*A1, *gyr* A96, *thi*, *hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *laq*I^{q}ZΔMB]. The cells were grown overnight in 5ml Luria-Bertani (LB) broth at 37°C. The overnight culture was diluted 1:1000 into 100ml of LB broth and incubated at 37°C until absorption reached 0.5OD at 550nm. The cells were chilled on ice for 10min in two pre-cooled 50ml centrifuge tubes before centrifugation at 4°C and at 4-6,000rpm for 5 min. The cells were then resuspended in 15ml of chilled 0.1M $MgCl_2$ solution before centrifugation at 4°C for 5min at 4-6,000rpm. The pellet was carefully resuspended in 15ml of chilled 0.1M $CaCl_2$ and incubated on ice for 20min before centrifugation at 4°C for 5min at 4-6,000g. The pellet was resuspended in 2.5ml of chilled $CaCl_2$ solution and 14% glycerol and stored as 200µl aliquots in microcentrifuge tubes. The cells were then frozen with liquid nitrogen and stored at -70°C until used for transformation.

2.22 Ligation of PCR DNA into pGEM®-T Easy cloning vector

A ligation mixture containing insert DNA (150ng), pGEM®-T Easy cloning vector (50ng) (Promega) (see appendix A11 for diagram of vector), T4 DNA ligase (3 Weiss units) (Promega), 1x ligation buffer (Promega) the DNA and vector were left to ligate overnight at 4°C.

2.23 Transformation of ligated DNA into competent JM109 cells

A 50µl aliquot of competent JM109 cells were thawed on ice. To 50µl of competent cells, 2.5µl of ligated DNA/vector was added. A control transformation containing circularised plasmid DNA was also set up to determine the competency of the cells. The cells were then left to incubate on ice for 10min before heat shock at 42°C for 45-

50s. After heat shock the cells were returned to ice for 2min before the addition of 900µl ice-cold SOC medium (see appendix A1). The transformation reaction was left to incubate for 60min at 37°C in a waterbath. The transformed cells were centrifuged and resuspended into 200µl of LB medium and spread out onto a LB agar plate (supplemented with 100µg/ml ampicillin, 0.1mM isopropyl-β-*D*-thiogalactopyranoside (IPTG) and 40µg/ml 5-bromo-4-chloro-3-indolyl-β-*D*-galactopyranoside (X-Gal)) for blue/ white colony screening (see appendix A1). The inoculated plates were incubated aerobically at 37°C for 12-14h.

The clones containing inserts were selected by blue/white colony screening. The addition of IPTG and X-Gal to the plates allowed the selection of clones that had a disrupted *lacZ* gene that contained the inserted DNA.

2.24 Small scale plasmid preparations

The white colonies were taken from the agar plates and used to inoculate 5ml LB broth containing 100µg of the antibiotic, ampicillin. The inoculated LB broth was incubated overnight in a shaking incubator at 37°C. The plasmid was extracted and purified from the overnight bacterial cultures by using the QIAprep Spin Plasmid Kit (Qiagen), according to the manufacturer's instructions.

2.25 Analysis of cloned DNA by restriction digest and electrophoresis

The analysis of the cloned fragment was achieved through separating the fragment from the cloning vector / plasmid by using restriction enzyme digestion and separation through agarose gel electrophoresis.

In a microcentrifuge tube, 4.8µl of the purified plasmid was digested in a 14µl reaction containing 6U *Eco*RI (Promega), 1x multi-core buffer (Promega) and incubated in a waterbath for 2h at 37°C. The digested products were then separated through gel electrophoresis with the appropriate DNA ladder on a 1% TAE agarose gel to verify that the cloned insert was of the correct size and orientation. Plasmids containing the correct sized fragments in the correct orientation were then sequenced using M13UNIV and M13REV primers (see appendix A10 for primer sequences). The gene

sequences were then further analysed through sequence alignments to measure the relatedness of the sequences.

2.26 BLASTN and BLASTX sequence analysis of cloned fragments.

To investigate the relatedness of the novel *Burkholderia* isolates and *Burkholderia* species 16S rDNA sequence data, a program capable of aligning and comparing numerous these sequences was required. The sequence data was edited (regions between the PCR primer sites were only used) and analysed using the GCG package of sequence programs (Devereaux *et al.*, 1984). For further analysis the nucleotide sequences were then changed into FASTA format using the UK HGMP sequence editor (http://www.hgmp.mrc.ac.uk/), then entered into the NCBI database (http://www.ncbi.nlm.nih.gov/) using the BLASTN and BLASTX program to find the sequences had any homology to published sequences of *Burkholderia* species and other bacteria.

2.27 Alignment of DNA sequence data using ClustalX and GeneDoc.

The sequence data once analysed was aligned with other sequence data to calculate the homology using the ClustalX program (Thompson *et al.*, 1997). The aligned sequence data would show any areas of heterogeneity which could be used to design species specific primers or calculate the relatedness of the sequences.

The construction of the final phylogenetic tree takes place in three stages. The sequences are compared to each other in a pairwise alignment, from this a dendrogram is constructed describing the approximate groupings of the sequences by similarity.

A final alignment is made using the dendrogram is a guide. From this final alignment is made using a Neighbour Joining (NJ) method by Saitou and Nei. This calculates the distance between all pairs of sequences. The NJ tree is then 'bootstrapped' to generate a confidence level for the groupings within the tree. This uses a random counting of how many different trees there are and the groupings that occur in a sample tree, this is replicated between 500-1000 times. The phylogenetic tree

generated from this program could then be viewed using the TreeView program (http://taxonomy.zoology.gla.ac.uk/rod/treeview/) (Page, 1996). Through the use of this program the phylogenetic tree can be rooted through the selection of the out-group sequence. This would produced a phylogram which would show the relationship between relatively unknown isolates and fully characterised and known species of bacteria.

Table 2.3 *Burkholderia* species and their origins with 16S rRNA gene sequence accession numbers

The table below contains the source and accession numbers of 16S rDNA gene sequences of *Burkholderia* species and partially characterised *Burkholderia* isolates contained within GenBank. The table also contains the details of where they were isolated or source, so any character specific clusters formed within the phylogram can be easy identified.

The sequence data for the *Burkholderia* species and other *Burkholderia* isolates were obtained through the nucleotide sequence Genbank database found on the Entrez website (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). The novel woodland isolates will have the 16S rDNA PCR carried out on them and the 16S rDNA used for further analyses.

Taxon and strain	Source	GenBank accession No.
New rhizospheric *B. cepacia* sp. isolates [a]		
SH1[b]	Leek field soil (Shifnell, Shropshire, UK)	AJ300685
SH13E	Woodland soil and leaf litter (Shropshire, UK)	AJ300686
K14	Potato soil (Egypt)	AJ300687
S512	Agricultural soil (Slough, UK)	AJ300688
13SM[c]	*Triticum aestivum* L. roots	AJ300689
OSR40	*Quercus* sp seedling roots (Cardiff, Wales)	AJ300690
RW4	*Pinus* sp. Rotting pine wood	AJ300691
RW36[c]	Rooting wood	AJ300692
WR19x	Woodland roots	AJ300693
GR34	*Senecio vulgaris* L. roots	AJ300694
RR8	*Allium cepa* L.	AJ300695
WF23	West Farm soil (UK)	AJ300698
OY715	*Allium cepa* L. (A supermarket- York, UK)	AJ300696

Taxon and strain	Source	GenBank accession No.
ON1[b]	*Allium cepa* L. (A supermarket – London, UK)	AJ300697
***B. cepacia* complex [d]**		
B. cepacia [I] ATCC[e] 25416	*Allium cepa* L. (USA)	U96927
NCPPB[f] 3025	*Allium cepa* L. (Italy)	AJ300684
B. multivorans [II] LMG 13010	CF patient (Belgium) Coeyne *et al.*, 2001b	AF148555
B. cepacia [III]LMG 12614	CF patient (UK) Balandreau *et al.*, 2001	AF311969
LMG 12615	CF patient (UK) Balandreau *et al.*, 2001	AF311970
LMG 16656	CF patient (UK) Coenye *et al.*, 2001b	AF48556
WS11.7 LMG 199232	*Triticum aestivum* L. shoots (Australia) Balandreau *et al.*, 2001	AF311971
LS 2.4 LMG 19233	Lupin shoots (Australia) Balandreau *et al.*, 2001	AF311972
C3BIM	*Graminse* sp. Maize rhizosphere (France) Viallard *et al.*, 1998	U96938
B. stabilis [IV] LMG 14294 [T]	CF patient (Belgium) Vandamme *et al.*, 2000	AF097533
B. vietnamiensis[V] LMG10929[T]	Rice rhizosphere, Vietnam, Kersters *et al.*, 1995	U96928
B. cepacia [VI] LMG 18941	CF patient (Belgium) Coeyne *et al.*, 2001b	AF175314
B. gladioli pv *gladioli* ATCC 10248 [T]	*Gladiolus* sp. (USA)	X67038
B. andropogonis LMG 2129 [T]	*Sorghum bicolour* L. Li *et al.*, 1993	X67037
B. plantarii LMG 9035 [T]	*Oryza sativa* (Japan)	U96933
B. glathei LMG 14190 [T]	Unknown (Germany)	U96935
B. graminis C4DIM [T]	*Graminae* sp. (France) Viallard *et al.*, 1998	U96939
B. pyrrocinia LMG 14191 [T]	Soil, Viallard *et al.*, 1998	U96930
B. caribensis MWAP71	Vertisol (Martinique, West Indies) Achouak *et al.*, 1999	Y17010
B. phenazinium LMG 2247 [T]	Soil, Viallard *et al.*, 1998	U96936
B. cocovenenans LMG 11626 [T]	Fermented coconut (Java) Van Damme *et al.*, 1960	U96934
B. pseudomallei 1026[b]	Environmental isolate, Brett *et al.*, 1997	U91839
B. thailandensis E264	Environmental isolate, Brett *et al.*, 1998	U91838
B. caryophylli LMG 2155 [T]	*Diathus caroyophillus*	X67039
B. kururiensis	Polluted water, Zhang *et al.*, 2000	AB024310
B. glumae CFBP [h] 2430 [T]	*Oryza sativa* (Japan)	U96931
B. graminis C3AIM	*Graminae* Maize rhizosphere (France)	Not entered
B. graminis C5AIM	*Graminae* Maize rhizosphere (France)	Not entered
B. graminis C4DIM	*Graminae* Maize rhizosphere (France)	Not entered
B. uboniae	Yabuuchi *et al.*, 2000 (France)	AB030584
B. vandii LMG 16020 [T]	Orchid rhizosphere	U96932
B. tuberum STM678	*Aspalathus camosa* L. (South Africa) Moulin *et al.*, 2001	AJ302311
B. phymatum STM815	*Machaerium lanatum* L. (French Guiana) Moulin *et al.*, 2001	AJ302312
B. anthina LMG 16670	*Carludoucas palmata* rhizosphere, UK Vandamme *et al.*, 2002b	AF456060
B. caledonica LMG 19076 [T]	Rhizosphere soil (Edinburgh, UK) Coeyne *et al.*, 2001c	AF215704
B. fungorum LMG 16225 [T]	White-rot fungus (*Phanerochaete chrysosporium*) Coeyne *et al.*, 2001c	AF215705
B. ambifaria LMG 19182[T]	Pea rhizosphere, Coeyne *et al.*, 2001d	AF043302
B. sacchari LMG 19450 [T]	Sugar cane plantation soil (Brazil) Bramer *et al.*, 2001	AF263278
B. brasilensis M130	Sugar cane, Magalhães Cruz *et al.*, 2001	AF49136

Taxon and strain	Source	GenBank accession No.
B. tropicalsis Ppe8	Magalhães Cruz *et al.*, 2001	AJ420332
B. kirkii 835462	Endosymbiont of leaf galls, *Psychotria*	AF4750689
B. hospita LMG20599	Soil rhizosphere, Goris *et al.*, 2002	AY040366
B. terricola LMG20594	Soil rhizosphere, Goris *et al.*, 2002	AY040362
Other unclassified *Burkholderia* sp. Of rhizospheric and environmental origin		
Burkholderia sp. LB400	Soil (USA) Fain and Haddock, 2001	U37343
Burkholderia sp. N3P2	Phenanthrene-enriched soil (USA) Mueller *et al.*, 1997	U37344
Burkholderia sp. N2P5	Phenanthrene-enriched soil (USA) Mueller *et al.*, 1997	U37342
Burkholderia sp. N2P6	Phenanthrene-enriched soil (USA) Mueller *et al.*, 1997	U37343
Burkholderia sp. CRE7	Phenanthrene-enriched soil (USA) Mueller *et al.*, 1997	U37340
Burkholderia sp. GSOY	Soy diesel (USA)	U16140
Burkholderia sp. VUN10013	Phenanthrene-enriched soil (Australia)	AF068011
Burkholderia sp. SAP II	Unknown (USA)	AF052387
Burkholderia sp. M35b	*Graminae*, Maize rhizosphere (France) Viallard *et al.*, 1998	U96937
Burkholderia sp. JB1	PCB[i]-enriched soil (Netherlands) Springael *et al, 1996*	X92188
Burkholderia sp. Dha-54	Dehydroabietic acid-enriched forest soil (Germany)	BSP011508
Burkholderia sp 2.2N casida	Clay soil (USA) Cain *et al.*, 2000	AF226727
Burkholderia sp. HY1	Aniline- enriched environment (USA)	AF210314
Burkholderia sp. YY62	T-buyl ester-enriched environment (Japan)	AB011287
Burkholderia sp. AB100	Unknown (South Korea)	AF219126
Burkholderia sp. AB2	Unknown (South Korea)	AF219125
Burkholderia sp. P18G1120	Soil (Germany) Schwieger & Tebbe, 2000	AF214131
Burkholderia sp. P18G1145	Soil (Germany) Schwieger & Tebbe, 2000	AF214132
Burkholderia sp. RP007	Phenanthrene-enriched soil (New Zealand)	AF061872
Burkholderia sp. KN-B3	3-chlorobenzoate-enriched soil (Netherlands)	AF074711
Burkholderia sp. KN-B9	3-chlorobenzoate-enriched soil (Netherlands)	AF074712
Burkholderia sp. 28094	*Graminae*, rhizosphere (Italy) Di Cello *et al.*, 1997	U91570
Burkholderia sp. KP24	Trichlorethylene-enriched soil (Japan) Hanada *et al.*, 1998	AB015606
Burkholderia sp. NAH1	Phananthrene-enriched soil (France)	AF247815
Burkholderia sp. JS150	Unknown (Canada)	AF262932
Burkholderia sp. IpA-51	Isopimaric acid –enriched soil (Germany)	BSP011509
Burkholderia sp. CAB-02	Unknown (Japan)	AB041730
Burkholderia sp. TH2	Benzoate-enriched soil (Japan)	AB041772
Burkholderia sp. S4.9	Phenanthrene-enriched soil (Germany) Friedrich *et a.*, 2000	AF247496
Burkholderia sp. NF100	Fenitrothion-enriched soil (Japan) Hayatsu *et al.*, 2000	AB025790
Burkholderia sp. TFD6	2,4-D-enriched soil (USA)	AF184931
Burkholderia sp. A6.2	Phenanthrene –enriched soil (Germany) Friedrich *et al.*, 2000	AF247491
Burkholderia sp. WD285	PCB-enriched soil (Germany) Nogales *et al.*, 2001	AJ292644
Burkholderia sp. WD263	PCB-enriched soil (Germany) Nogales *et al.*, 2001	AJ292641
Burkholderia sp. WD227	PCB-enriched soil (Germany) Nogales *et al.*, 2001	AJ292639
Burkholderia sp. WD289	PCB-enriched soil (Germany) Nogales *et al.*, 2001	AJ292645
Burkholderia sp. WD206	PCB-enriched soil (Germany) Nogales *et al.*, 2001	AJ292643
Burkholderia sp. WD202	PCB-enriched soil (Germany) Nogales *et al.*, 2001	AJ292638
Burkholderia sp. WD2114	PCB-enriched soil (Germany) Nogales *et al.*, 2001	AJ292647

Taxon and strain	Source	GenBank accession No.
Burkholderia sp. WD2116	PCB-enriched soil (Germany) Nogales *et al.*, 2001	AJ292646
Other genera		
Ralstonia solanacearum		X67036
R. eutropa		RECH3416S
R. picketti		X67042
Pseudomonas aeruginosa		PA276651
Pandoraea apista LMG 16407^T	CF patient (Denmark) Coenye *et al.*, 2000	AF139173
P. norimbergensis LMG 18379^T	Sediment (Germany) Coenye *et al.*, 2000	Y09879
P. pulmonicola LMG 18106^T	CF patient (Canada) Coenye *et al.*, 2000	AF139175
P. pnomenusa LMG 18087 ^{T}	CF patient (UK) Coenye *et al.*, 2000	AF139174
P. sputorum LMG 18819 ^{T}	CF patient (USA) Coenye *et al.*, 2000	AF139176
Staphylococcus aureus	Unknown, Ludwig *et al.*, 1992	X68417

a. All new rhizospheric UK *Burkholderia* isolates were collected in and around Sand Hutton, York, UK and London, UK; Strains were isolated on PCAT, **b.** TB-T or **c.** SMSA media; **d.** *B. cepacia* complex genomovar numbers are shown in square brackets; **e.** ATCC, American Type Culture Collection, Manassas, VA, USA; **T** type strain; **f.** NCPPB, National Collection of Plant Pathogenic Bacteria, Central Science Laboratory York, UK; **g.** LMG, Laboratorium Microbiologie Gent Culture Collection, Gent, Belgium; **h.** CFBP, Collection Française de Bactéries Phytopathogènes, Institut National Recherche Agronomique, Station de Pathologie Végétale et Phytobactériologie, Angers, France; **i.** PCB, polychlorinated biphenyl.

2.28 DNA-DNA hybridisation *via* the micro-titre plate method

To assess the relatedness of two different DNAs, the kinetics of single stranded DNA-DNA binding is a good indicator of homology. The faster and stronger the two DNAs bind, the more related / homologous they are to each other (Ezaki *et al.*, 1989 and Adnan *et al.*, 1993).

2.28.1 DNA Fixation onto microtitre plates.

The genomic DNA for this assay was prepared through the use of the Genomic DNA Purification Wizard (Promega) according to the manufacturer's instructions.

The DNA was diluted with 0.1 X SSC (see appendix A2) to OD_{260} = 1, denatured at 100°C for 10min and cooled rapidly on ice. The DNA was then further diluted with PBS-Mg (see appendix A8) to OD_{260} = 0.25, 100µl of each diluted DNA solution was dispensed to each respective well of a flat bottom black microtitre plate (Maxi-sorp) (Nalgene-Nunc). The microtitre plate was then sealed with vinyl tape (Sigma-Aldrich) and the DNA was then left to bind at 30°C for 4h in a hybridisation oven. The DNA solution was removed and the bound DNA washed once with 300µl of 1X PBS (see appendix A8) and dried at 45°C for 30min. The plates were used for hybridisation within 24h.

2.28.2 Labelling probe DNA with photobiotin.

In an eppendorf, 2µg of genomic DNA was added to 10µl photobiotin solution (Sigma-Aldrich) (see appendix A8) and linked through exposure under a 400 Watt mercury lamp for 30 min on an aluminium block on ice. To the photobiotin - DNA solution, 185µl of 0.1M Tris-HCl was added, mixed thoroughly via vortexing and washed twice with 200µl Tris-HCl saturated butan-1-ol to remove any unlinked photobiotin. The unlinked photobiotin was removed with the upper solvent layer. The probe DNA was then sonicated for 30 s and denatured at 100°C for 10min and cooled rapidly on ice; 50µl of probe was required for every 1ml of hybridisation solution (see appendix A8) used.

2.28.3 DNA-DNA hybridisation / binding assay.

The DNA coated wells were filled with 200µl pre-hybridisation solution (see appendix A8) and left to pre-hybridise at 50°C for 40 min in a hybridisation oven.

The pre-hybridisation solution was replaced with 100µl hybridisation solution (see appendix A8) containing labelled probe DNA and left to hybridise for 50°C for 3h. After incubation the wells were washed three times with 300µl of 1X SSC. Streptavidin-β-galactosidase (ICN Inc.) solution (see appendix A8) was then added to each well and incubated at 37°C for 10min.

After incubation the streptavidin-β-galactosidase solution was removed and the wells washed three times with 300µl of 1X SSC. Finally, 100µl of 4-methylumelliferyl-β-D-galactopyranoside (Sigma-Aldrich) (4-MUG) solution (see appendix A8) was added to each well and the kinetic readings of the DNA binding were then taken every 15min for 2h at 37°C.

The fluorescent intensity of the bound probe DNA was measured with a Spectra Max Gemini XS platereader (Molecular Devices) using wavelengths of 360nm excitation, 450nm emission. A cut-off filter at 435nm was used to prevent excitation light crossover. The readings from the kinetic binding assay were recorded using the SoftMAX Pro software (Molecular Devices).

2.29 Bioremediative potential of novel *Burkholderia* isolates

It has been demonstrated that isolates from the genus *Burkholderia* have the ability to utilise a wide range of nutrients. Several isolates of *Burkholderia* have already been found to possess bioremediative properties, it is hoped that some of the novel isolates may also possess the ability to breakdown complex chemical compounds.

2.30 Bioremediation of polyaromatic hydrocarbons (PAHs) pyrene and phenanthrene by novel *Burkholderia* isolates.

Selected novel isolates of *Burkholderia* were used to investigate their bioremediative potential *in vitro* against a range of polyaromatic hydrocarbons (PAHs). The novel *Burkholderia* isolates selected for this investigation were those which were closely related to known bioremedial isolates of *Burkholderia* within the 16S rDNA phylogram. It is hoped that isolate selection based on this criterion would result in the detection of bioremediation of PAHs in these novel isolates.

Single solutions of PAHs were used, a mix may have contained a PAH within it which had an inhibitory effect on the bacterial growth. However, using a mix PAH solution may have demonstrated the ability of some *Burkholderia* isolates to break down a number of complex chemicals not just one.

The PAHs used in this investigation were phenanthrene and pyrene solutions (QMX Laboratories). Alongside the single PAHs, a mix (PAH No. 9 mix) containing sixteen different PAHs (Acenapththene, acenaphthylene, anthracene, benzo(a)anthracene, Benzo(b)flouranthene, Benzo(k)flouranthene, benzo(g,h,I)perylene, benzo(a)pyrene, chrysene, dibenz(a,h)anthracene, flouranthene, flourene, indeno(1,2,3,c,d)pyrene, napthalene, phenanthrene and pyrene) (QMX Laboratories) was also used.

2.31 PAH mix 9, pyrene and phenanthrene agar plates

The novel *Burkholderia* isolates were initially screened through solid media where the PAH's were incorporated into the minimal mineral / MM2 agar (see appendix A1). The molten MM2 agar was cooled to 55°C and supplemented with PAHs in cyclohexane to a final concentration of 250ng/ml. All PAH supplement agar plates were kept in the dark and used within a 24h period, to prevent PAH degradation due to exposure to UV light.

The lack of carbohydrates and the presence of PAH within the MM2 agar provide no other carbon source for the *Burkholderia* to use for growth. Utilisation of the carbon from the PAHs indicated that the PAHs within the MM2 agar may have been partially or completely broken down and used as an energy source by the *Burkholderia*.

The plates were inoculated with 10µl of a bacterial broth (10^3cfu/ml) dried then incubated at 28°C aerobically for 7-14 days. The following four conditions were set up PAH enriched, PAH supplemented with 0.2% glucose, 0.2% glucose and MM2 agar without glucose and PAH. These conditions covered the possibility of sole carbon usage of the PAH or co-metabolism in the presence of glucose. The concentrations of the PAHs within the agar matrix were 250ng/ml. The amount of growth was determined visually at 7d and 14d with the glucose supplement plate as the positive growth control. The *Burkholderia* isolates displaying strong growth were subsequently

grown on PAH supplemented agar (250ng/ml) in preparation for the liquid enrichment cultures.

2.32 Liquid enrichment cultures using pyrene and phenanthrene

Once the ability to grow on minimal media supplemented with PAH was noted, it was necessary to identify the metabolites formed during the process of bioremediation.

The solid PAH (Sigma-Aldrich) were dissolved in diethyl ether (BDH) to a working solution of 250ng/ml, 1ml of this of was used to coat sterile glass ballotini (Jencons) beads within a glass conical flask. The diethyl ether was evaporated at RT°C under a fume cupboard to leave the dry beads and PAH residue within the conical flask. To the PAH covered beads, 20ml of sterile MM2 broth was added and inoculated with 100µl of a 10^6 cfu /ml bacterial suspension (in MM2 broth).

Five bacterial conditions were used, PAH, PAH with 0.2% glucose, MM2 and MM2 with 0.2% glucose. A chemical control was also included to monitor the effects of evaporation of diethyl ether spiked with PAH at RT°C, after which MM2 media was added to it incubated at 28°C but was not inoculated with the bacterial suspension. The flasks were then incubated in the dark at 25°C on a rotation platform at 150rpm for 7 days.

After the period of incubation the enrichment cultures were sacrificed in order to extract the PAH from the aqueous solution. This was achieved through centrifugation of the bacterial broth to remove the organic matter. The supernatant was then removed and placed in a falcon tube and to this 20ml of AnalaR grade cyclohexane (BDH) was added. The two liquids were then shaken vigorously for 5min to form a fine emulsion, this ensured that the solvent had maximum contact with the aqueous phase in order to extract the PAH from it. The emulsion was then briefly centrifuged and the upper solvent phase was removed. The extraction stage was repeated twice so as to ensure maximum extraction of the PAH from the aqueous phase.

The cyclohexane fraction containing the PAH was placed in a glass flask to be evaporated under a vacuum at 40°C. The residual PAH was then resuspended in 2ml

of cyclohexane and stored in an amber glass vial for gas chromatography – mass spectrum (GC-MS) analysis (protocol used is confidential).

CHAPTER 3

ISOLATES AND PHENOTYPIC CHARACTERISATION OF NOVEL *BURKHOLDERIA* ISOLATES

3.0 Background

This chapter will aim to address the following hypotheses:

- *There are common phenotypic characteristics present in Burkholderia isolates found in woodland habitats*
- *Novel woodland Burkholderia isolates can be clustered according to species of tree or plant*

To achieve the objectives of these hypotheses, environmental sampling was initiated to obtain novel *Burkholderia* isolates from known plants and habitats.

3.1 Environmental sampling- Autumn 1999 Sand Hutton, York, UK

Environmental sampling was carried out on two separate occasions between September – November 1999 from two sites within the Sand Hutton area in York, United Kingdom. Samples were taken on a pseudo-random basis by selecting areas which would have a higher probability of obtaining samples containing *Burkholderia*. Environmental sampling considered the probability of obtaining *Burkholderia* isolates from a type of sample (avoiding very dry / exposed areas). The aim was to obtain a high number of *Burkholderia* isolates from the majority of the samples and to provide a large number of isolates from a diverse selection of sample types.

The following tables will show the number of *Burkholderia*-like colonies found from the various samples taken from the woodland sites. The suspected *Burkholderia*-like colonies were identified on PCAT agar as small cream or white domed shaped colonies. Based on this physical description, the numbers of *Burkholderia*-like colonies found on the PCAT agar were noted on the following table (and on subsequent tables) along with the origins of the sample. The colonies were examined further through Gram staining, cytochrome oxidase testing and *Burkholderia*- specific PCR analyses, all of these tests will be described in the following sections. Colonies taken from each PCAT plate for subsequent testing was not noted at the time of this study.

Table 3.1 Environmental sampling- Autumn 1999 Sand Hutton, York, UK

The serial dilutions from the samples were plated onto labelled PCAT agar plates as to enable the sample to be traced back (*eg* 1A- Area 1, pine – rotting wood). After incubation each agar plate was examined for the presence of *Burkholderia*-like colonies. Not all of the colonies from these plates were subcultured for further testing due to either fungal contamination or to too many colonies present. When colony numbers were higher than 20, a selection of up to 20 colonies were taken; the selection was based on ability to obtain 'clean' cultures. Eighty colonies were tested using the *Burkholderia* specific PCR analysis, 13 produced the 770bp amplicon confirming their *Burkholderia* genus status.

Agar Plate Name	Sample Description	Number of '*Burkholderia*-like' colonies	Number of confirmed *Burkholderia*
Sample Area 1			
A	*Pinus* sp. Pine-rotting wood	0	0
B	Unknown flowering plant-roots	0	0
C	*Quercus* sp. Oak-rotting wood	42	2
D	*Salix* sp. Willow-rhizospheric soil	0	0
E	*Fraxinus* sp. Ash-rotting wood	5	2
F	*Quercus* sp. Oak-rhizospheric soil	+60	2
G	Unknown fungus- rhizospheric	0	0
H	*Pinus* sp. Pine-rhizospheric soil	0	0
I	*Pinus* sp. Pine & Bramble- top soil	0	0
J	*Polypodiaceae* sp Fern-rhizospheric soil	1	0
Sample Area 2			
A	*Acer* sp. Sycamore-rotting wood	2	1
B	*Pinus* sp. Pine-surface litter	7	0
C	*Laurus* sp. Laurel sapling-roots	17	4
D	Unknown-rotting wood and moss	0	0
E	*Quercus* sp. Oak-rhizospheric soil	1	0
F	*Acer* sp. Sycamore-rhizospheric soil	20	1
G	*Laurus* sp. Laurel- leaf litter	5	1
Total		**+160**	**13**

3.1.1 Description of sampling areas

The environmental samples taken between September - November 1999 from two woodland sites in Sand Hutton, York, United Kingdom.

3.1.1.1 Area 1 - Bramble Woodland

The soil in this woodland was very moist due to the high clay content and contained very little plant matter within it; the leaf litter on top was very damp. The samples from this woodland were taken within 24h of rainfall. It is located near agricultural fields; the woodland floor was covered with low growing plants and shrubs with very little light reaching the ground due to a heavy tree canopy. The number of '*Burkholderia*-like' colonies recovered from this area were in excess of 108 from 10 environmental samples.

3.1.1.2 Area 2 – Open Woodland (clear floor)

The soil in this woodland had a high content of plant matter making it light and more aerated than the bramble woodland. There was a lot of leaf litter found on top of the soil and underneath the soil was relatively dry and crumbly. The woodland floor was clear with few low growing plants and shrubs, there was a good amount of light coming through the tree canopy. Samples were taken within 24h of rainfall, 52 '*Burkholderia*-like' colonies recovered from 7 environmental samples taken from this area.

3.1.2 *Burkholderia* specific PCR analysis from Autumn 1999 environmental sampling, York, UK

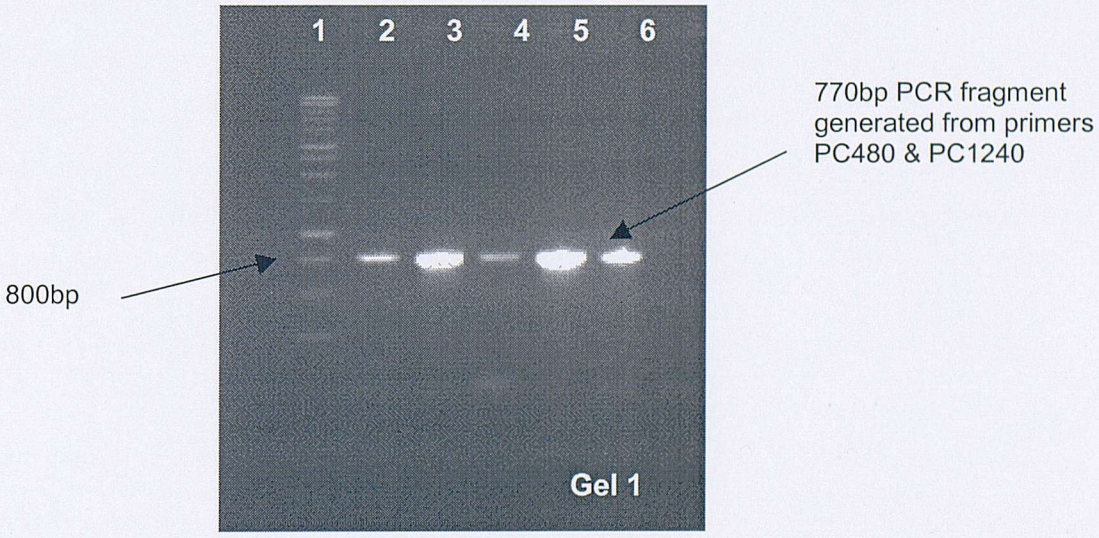
The '*Burkholderia*-like' colonies were observed and isolated from each sample plate then subcultured into nutrient agar and incubated at 28°C for 24h. After the incubation period the colonies were further characterised with Gram stain, cytochrome oxidase test before the *Burkholderia*-specific PCR analysis was carried out on isolates which were Gram negative and cytochrome oxidase positive. However, some *Burkholderia* are known to be cytochrome oxidase variable, if a colony tested cytochrome oxidase negative and after microscopic analysis appeared to be a Gram negative rod, it was included in the PCR analysis for confirmation. (results of Gram staining and cytochrome oxidase tests not included in this thesis). Due to the high number of '*Burkholderia*-like' colonies on some of the PCAT agar plates, the selection of colonies based on ease of isolation (not obviously in contact with other colonies or fungal growths) was implemented. The colonies were then subcultured onto nutrient agar plates, incubated and then checked for purity (see appendix A3 for streaking method). Any mixed cultures were subcultured again to obtain pure cultures for testing. Should the separated cultures appear to be more than one type *Burkholderia*-like colonies;

they were each checked with Gram staining, microscopic analysis and cytochrome oxidase test before PCR analysis.

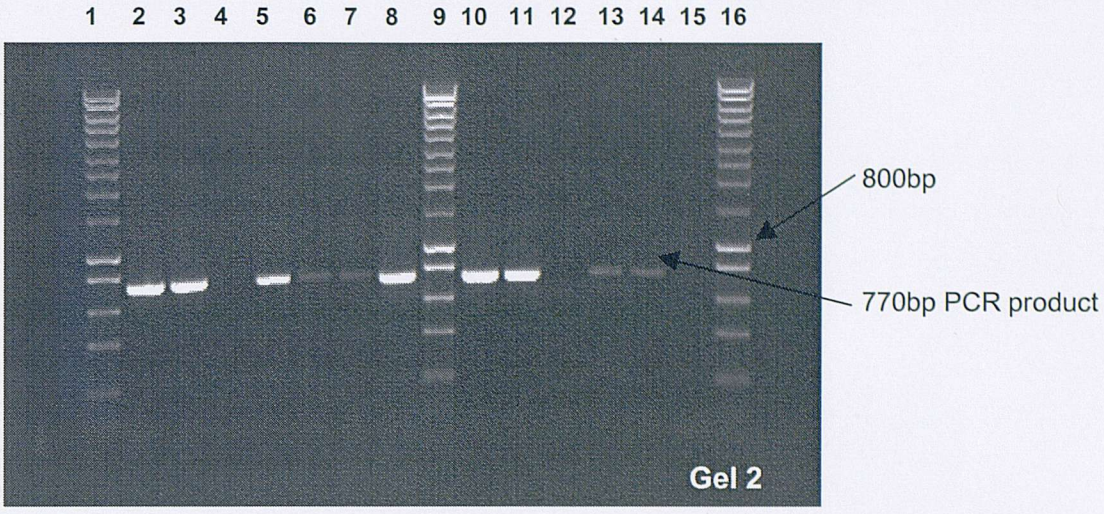
The presence of a 770bp PCR product (see figure 3.1) indicated that the isolate in question was a member of the genus *Burkholderia*. Of the 63 isolates selected and tested from the two sample areas, 13 proved to be confirmed as *Burkholderia* by this PCR analysis. Once confirmed as *Burkholderia*, the samples were allocated code identifications for the novel isolate library. These codes allowed the isolates to be trace-backed to the original sample material (see appendix A7 for coding system for isolates). All isolates testing confirmed as *Burkholderia* were included for further phenotypical characterisation through fatty acid analysis, carbohydrate and protein profiling.

Figure 3.1 *Burkholderia* specific PCR amplification, Autumn 1999

The gel images display the 770bp PCR fragment produced by the environmental *Burkholderia*-like isolates. Lane 4 on Gel 2 shows an isolate that failed to produce a 770bp fragment; the isolate was discarded and not placed forward for further characterisation.



Lane 1 Marker Hyper Ladder I (Bioline), Lane 2 Laurel (*Laurus* sp.) root, Lane 3 Laurel (*Laurus* sp.) root, Lane 4 Laurel (*Laurus* sp.) root, Lane 5 Oak (*Quercus* sp.) rotting wood, Lane 6 Oak (*Quercus* sp.) rotting wood.



Lane 1 Marker-HyperLadder I, Lane 2 Sycamore (*Acer* sp.) rotting wood, Lane 3 Laurel (*Laurus* sp.) root, Lane 4 Oak (*Quercus* sp.)- Rotting wood Lane 5 Fern (*Pteridium aquilinum*) rhizospheric soil, Lane 6 Oak (*Quercus* sp.) rhizospheric soil, Lane 7 Oak (*Quercus* sp.) rhizospheric soil, Lane 8 Sycamore (*Acer* sp.) rhizospheric soil , Lane 9 Marker Hyper Ladder I, Lane 10 Sycamore (*Acer* sp.) rotting wood, Lane 11 Pine (*Pinus* sp.) leaf litter, Lane 12 Negative *Burkholderia* control -*Escherichia coli* JM109, Lane 13 Unknown plant root, Lane 14 Positive *Burkholderia* control *B. cepacia* type strain 2993, Lane 15 Negative PCR control, Lane 16 Marker- Hyper Ladder I.

3.2 Environmental sampling- Spring 2000, woodland farmland / agricultural land, York, UK

The areas were sampled on three occasions during February – April 2000 were from woodland sites in Sand Hutton, York and agricultural fields around York. The agricultural samples were taken to investigate the prevalence of *Burkholderia* within agricultural soils compared with woodland soils. The agricultural fields sampled were already harvested and ploughed, therefore, rhizospheric soil samples could not be obtained from this particular area.

Table 3.2 Environmental sampling- Spring 2000, woodland and farmland / agricultural land, York, UK

The samples from these three areas generated 103 *Burkholderia*-like colonies, 45 were selected on the basis of ease of isolation and obtaining a pure culture. The two agricultural sampling areas (Areas 3 and 4) failed to produce any *Burkholderia*-like colonies.

Agar Plate Name	Sample- Description	Number of *Burkholderia*- like' colonies	Number of confirmed *Burkholderia*
Sampling Area 2			
A	*Fagus* sp. Beech-rhizospheric soil- roadside	16	3
B	*Ulmus* sp. Elm-rhizospheric soil	9	2
C	*Fraxinus* sp. Ash-rhizospheric soil	3	0
D	*Betula pendula* Silver birch-rhizospheric soil	15	1
E	*Pinus sylvestris* Scots pine-rhizospheric soil	13	0
F	*Betula pendula* Silver-birch rhizospheric soil	19	2
G	*Quercus* sp. Oak-rhizospheric soil	28	3
Sampling Area 3			
	Hordeum vulgere -Barley field-rhizospheric soil	0	0
Sampling Area 4			
	Hordeum vulgere- Barely field-rhizospheric soil	0	0
Total		**103**	**11**

3.2.1 Description of sampling areas

Woodland sites sampled during this period were the same sites sampled in Autumn 1999. The agricultural soil samples were included to compare the *Burkholderia* isolates obtained from the woodland sites with those from the agricultural sites.

3.2.1.1 Area 3- Langlands Farm, East Yorkshire- Barley field- Dense and clay-type soil.

Dense heavy soil with a high moisture content; the field was surrounded by other arable fields. When the soil was mixed with water, the aqueous section appeared to be very cloudy showing that the soil had a fine particle matrix.

Very little plant matter was found floating near the top with most of the soil sinking to the bottom of the bottle.

3.2.1.2 Area 4- Unknown Town centre, East Yorkshire - Barley field- Clay soil.

Dense heavy soil with a high moisture and clay content; the field was in near vicinity of a town centre. When the soil was mixed with water and shaken, the aqueous section appeared to be very cloudy showing that the soil had a fine particle matrix. Very little plant matter was found floating near the top with most of the soil sinking to the bottom of the bottle.

3.2.2 *Burkholderia* specific PCR analysis of the colonies isolated – Spring, 2000

Out of the 103 '*Burkholderia* type' colonies observed from the sampling of areas 2, 3 and 4, 45 colonies were subcultured and subsequently analysed using *Burkholderia* specific PCR amplification. Through the PCR amplification analysis, 11 of the 45 isolates tested were found to be from the genus *Burkholderia*.

3.3 Environmental sampling- Summer 2000, Hampstead Heath (woodlands), UK

The environmental sampling took place on three occasions between May-June 2000 in woodland areas within Hampstead Heath, London, United Kingdom. The serial dilutions of the samples were plated out onto labelled PCAT plates, which enabled trace-back to the original environmental sample.

Table 3.3 Environmental sampling- Summer 2000, Hampstead Heath

After incubation the PCAT plates were examined for the presence of *Burkholderia*-like colonies, the numbers on each plate were noted.

Plate Name	Sample description	Number of '*Burkholderia*-like' colonies	Number of confirmed *Burkholderia*
Sample Area 5 Spring 2000			
A	*Quercus* sp. Oak- rhizospheric soil	15	2
B	*Quercus* sp. Oak- leaf litter	2	0
C	*Betula* sp. Birch- rhizospheric soil	9	1
D	*Taxus baccata* Yew- leaf litter	0	0
E	*Quercus* sp. Oak- rhizospheric soil	19	4
F	*Fagus* sp. Beech rotting wood	11	0

Plate Name	Sample description	Number of '*Burkholderia*-like' colonies	Number of confirmed *Burkholderia*
D	*Taxus baccata* Yew- leaf litter	0	0
E	*Quercus* sp. Oak- rhizospheric soil	19	4
F	*Fagus* sp. Beech rotting wood	11	0
G	*Fagus* sp. Beech- rhizospheric soil	17	2
H	*Fagus* sp. Beech- rhizospheric soil	10	0
I	*Salix* sp. Willow- rhizospheric soil	9	1
J	*Aesculus hippocastanum* Horse chestnut- rhizospheric soil	9	0
K	*Acer* sp. Sycamore- rhizospheric soil	14	3
L	*Quercus* sp. Oak- rhizospheric soil	10	0
M	*Taxus baccata* Yew- rhizospheric soil	2	0
N	*Quercus* sp. Oak- rhizospheric soil	10	0
O	Unknown rotting wood	18	1
P	*Betula pendula* Silver birch- rhizospheric soil	21	5
R	*Quercus* sp. Oak leaf litter	5	0
S	*Pinus* sp. Pine- rhizospheric soil	4	1
T	*Fagus* sp. Beech- rhizospheric soil	20	0
Sample Area 6 Summer 2000			
A	*Quercus* sp. Oak- rhizospheric soil	6	2
B	*Fagus* sp. Beech- rhizospheric soil	1	1
C	Unknown plant root	4	0
D	*Betula sp.* Birch- rhizospheric soil	2	0
Total		**205**	**23**

From these two sampling areas, 205 *Burkholderia*-like colonies were observed on the PCAT plates.

3.3.1 Description of the sampling areas

During this sampling period, several small woodland sites within the Hampstead Heath area were sampled. The sampling areas were re-visited on three occasions to gain a degree of repetition in the samples obtained.

3.3.1.1 Area 5- Woodland area in Hampstead Heath, London, UK.

The soil had a high plant material content with large soil particles which sank to the bottom of the water. The aqueous section appeared almost clear indicating low clay content. The soil appeared to be well aerated; the woodland was open and light was able to reach the woodland floor. The layer of leaf litter (approximately 5 cm deep) on top of the soil seemed to keep the soil underneath cool and helping to retain some water. The samples were taken within 24h of rainfall occurring (table 3.3).

3.3.1.2 Area 6- Landscaped area in Kensington Gardens, London, UK.

The soil had some plant matter with most of the soil sinking to the bottom. The soil felt dry to touch, loamy and well aerated, the samples were taken a few days after rainfall (table 3.3). The soil may have been exposed to heat from sunlight, as leaf litter was minimal in this area; the samples were taken during a week long dry period in the summer months.

3.3.2. *Burkholderia* specific PCR analysis of the colonies isolated – Summer, 2000

Out of the 205 *Burkholderia* type colonies observed on the PCAT agar plates, 81 colonies selected and were sub-cultured to check for purity. The pure cultures were then further characterised using the *Burkholderia*-specific PCR analysis. The *Burkholderia* specific PCR analysis confirmed that 23 of these colonies belonged to the genus *Burkholderia*.

3.4 Environmental sampling - Hampstead Heath, Summer 2001, Hampstead Heath, Area 5 and 6

The sampling areas within Hampstead Heath (areas 5 and 6) were revisited to obtain further environmental samples (refer to sections 3.3.1.1 and 3.3.1.1 for descriptions of the areas). The numbers of *Burkholderia*-like isolates can be found on the table 3.4

Table 3.4 Environmental sampling - Hampstead Heath, Summer 2001

A number of environmental samples were obtained from this sampling area on two occasions. After incubation the number of *Burkholderia*-like colonies were observed and the numbers on each PCAT plate recorded.

Plate Name	Sample description	Number of '*Burkholderia*-like' colonies	Number of confirmed *Burkholderia*
Sample Area 5 (first visit)			
A	*Fagus* sp. Beech-rhizospheric soil	12	3
B	*Acer* sp. Sycamore leaf litter	6	0
C	*Quercus* sp. Oak-rhizospheric soil	15	4
D	*Quercus* sp. Oak leaf litter	10	0
E	*Acer* sp. Sycamore-rhizospheric	15	3

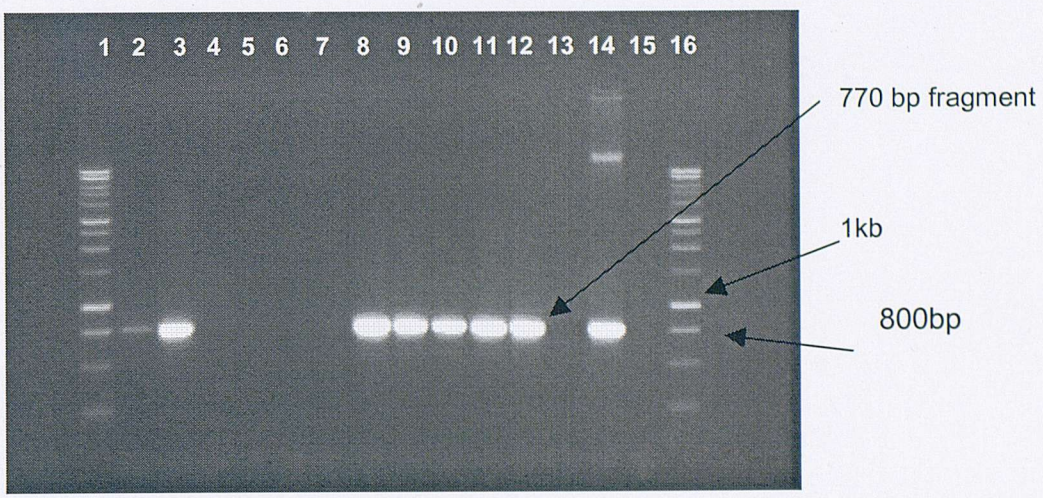
Plate Name	Sample description	Number of '*Burkholderia*-like' colonies	Number of confirmed *Burkholderia*
	soil		
F	Unknown flowering plant root	10	1
G	*Betula* sp. Birch-rhizospheric soil	7	5
H	*Fraxinus* sp. Ash-rhizospheric soil	6	1
I	*Betula* sp. Birch-rhizospheric soil	3	0
J	*Betula* sp. Birch leaf litter	6	2
K	*Quercus* sp. Oak-rhizospheric soil	3	0
L	*Ptilium* sp. Moss on *Fagus sp.* beech tree trunk	4	2
Sample Area 5 (second visit)			
A	*Fagus* sp. Beech-rhizospheric soil	9	2
B	*Ptilium* sp. Moss on *Fagus* sp. beech tree trunk	8	3
C	*Betula pendula* Silver birch leaf litter	2	0
D	*Betula pendula* Silver birch-rhizospheric soil	13	0
E	*Quercus* sp. Oak-rhizospheric soil	6	3
F	*Quercus* sp. Oak leaf litter	0	0
G	*Ptilium* sp. Moss on *Quercus sp.* oak tree trunk	11	4
H	*Quercus* sp. Oak-rhizospheric soil	6	5
I	*Quercus* sp. Oak leaf litter	0	0
Total		**152**	**38**

3.4.1. *Burkholderia* specific PCR analysis of colonies isolated-Summer 2001

Out of 152 '*Burkholderia*-like' colonies isolated from the PCAT agar plates, 70 colonies were selected and tested with the *Burkholderia* specific PCR amplification analysis (table 3.4). The analysis confirmed that 38 of these isolates were from the genus *Burkholderia* (figure 3.2). Once the isolates were confirmed as *Burkholderia* they were nominated a new identification code for the novel *Burkholderia* library.

Figure 3.2 *Burkholderia* specific PCR amplification of environmental isolates*, Summer 2001, Hampstead Heath, UK

Lanes 4, 5, 6, 7 and 13 showed that the sampled failed to generate the 770bp amplicon indicative of an isolate belonging to the genus *Burkholderia*. These isolates were not put forward for further analysis or characterisation and were discarded.



Lane 1 1kb Marker (Promega) Lane 2, 3, 4 Sycamore (*Acer* sp.) rhizospheric soil isolate a, b and c; Lane 5, 6, 7 Sycamore (*Acer* sp.) tree leaf litter a, b, and c; Lane 8, 9, 10 Oak (*Quercus* sp.) rhizospheric soil a, b, and c; Lane 11, 12, Beech (*Fagus* sp.) rhizospheric soil a and b; Lane 14, Positive control *B. cepacia* NCPPB 2993 type strain; Lane 15 Negative control, Lane 16 1kb Marker

***Isolates were later allocated codes for novel isolates library and stored at -80°C.**

3.5 Environmental sampling- Sand Hutton, York, UK – Autumn 2001

Sample areas 1 and 2 were revisited and samples taken to isolate and analyse any novel *Burkholderia* isolates (refer to sections 3.2.1 and 3.2.2 for descriptions of sample areas) (Table 3.5). Out of the 36 *Burkholderia*-like isolates, all 36 colonies were selected, subcultured to check for purity and tested by *Burkholderia*-specific PCR amplification. The results of the PCR amplification showed that 11 of these isolates were from the *Burkholderia* genus (gel image not available). The isolates which generated the required 770bp amplicon were allocated code identifications for the novel isolates library (see appendix A7 for coding system).

Table 3.5 Environmental sampling- Sand Hutton, York, UK – Autumn 2001

The woodland sites sampled previously were revisited to obtain further environmental samples. Sampling took place on two occasions between October and November 2001.

Plate Name	Sample description	Number of '*Burkholderia*-like' colonies	Number of confirmed *Burkholderia*
Sample Area 1			
A	*Trifolium* sp. Clover root	0	0
B	*Anthrisus sylvestris*- Cow parsley root	0	0
Sample Area 2			
A	*Laurus* sp. Laurel plant root	3	0
B	*Vaccinium myrtillus L.* Bilberry root	12	5
C	*Acer* sp. Sycamore rhizospheric soil	4	1
D	*Quercus* sp. Oak rhizospheric soil	4	2
E	*Quercus* sp. Oak rhizospheric soil	2	0
F	*Betula pendula* Silver birch rhizospheric soil	9	3
G	*Betula pendula* Silver birch rhizospheric soil	2	0
Sample Area 8			
A	*Salix* sp. Willow rhizospheric soil	0	0
Total		**36**	**11**

3.5.1 Description of sample area

The sites sampled during this period were the same sites previously visited in autumn 1999 (See sections 3.1.1.1 and 3.1.1.2 for descriptions of areas 1 and 2).

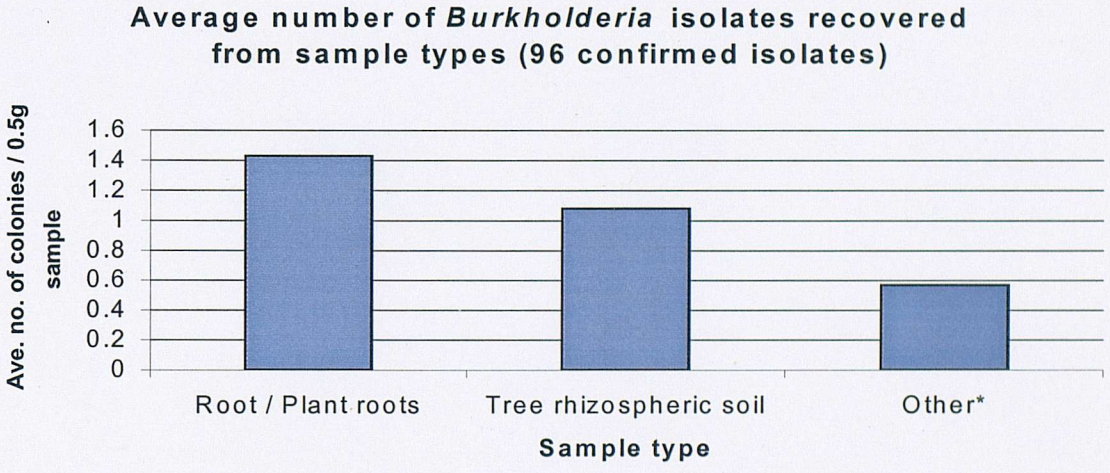
3.5.1.1. Area 8 – Landscaped land- Central Science Laboratory grounds, Sand Hutton, York, UK.

This was a landscaped area within the Central Science Laboratories grounds, the willow tree sampled was within 5m of a lake. The soil was particularly wet, heavy, had a high clay content and dense. Plant matter was relatively high in this soil sample, this was due to the use for decorative wood chippings on the landscaped gardens.

3.6 Prevalence within environmental samples and seasonality of *Burkholderia*

To measure the prevalence within environmental samples and the seasonality of *Burkholderia*, the numbers of confirmed *Burkholderia* isolates were noted at each sampling period. The average numbers per sample were used to investigate whether the prevalence of *Burkholderia* was found within the environment was constant or whether it fluctuated. The prevalence of *Burkholderia* within sample types was also measured to investigate whether *Burkholderia* was found in higher concentrations within certain samples. The results of the sampling data collected can be found on the graphs below.

Figure 3.3 The prevalence of *Burkholderia* isolate recovery from different sample types

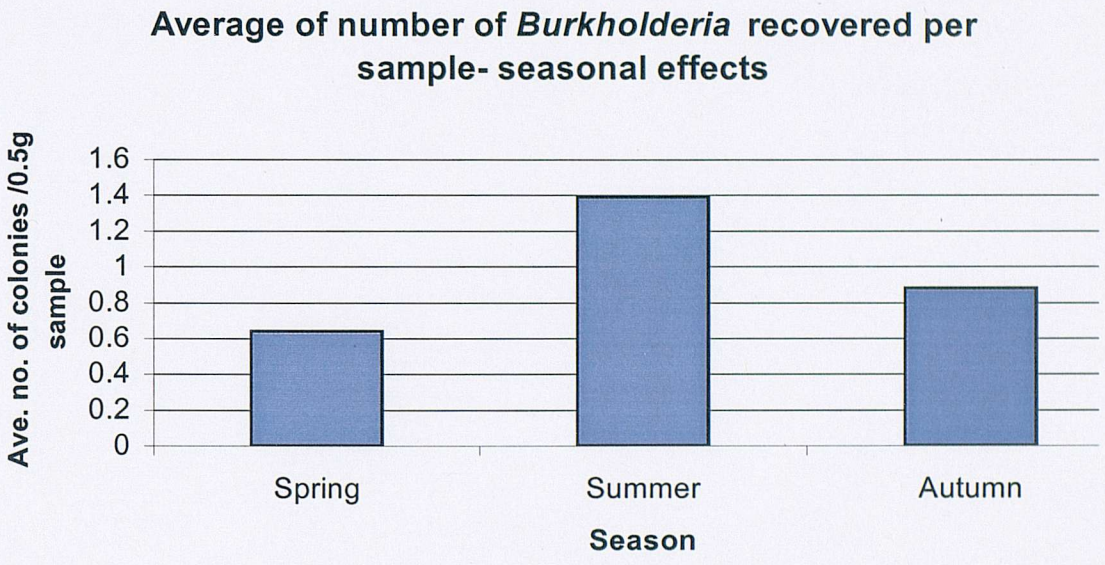


* Includes samples such as leaf litter and rotting wood.

The graph above represents the average number of *Burkholderia* confirmed isolates found within each environmental sample. The samples were classed into three groups, roots, rhizospheric soil and other. There were a total of 96 environmental samples confirmed as *Burkholderia* during the course of this study. The number of confirmed *Burkholderia* isolates were divided by the number of samples taken (*i.e. x* colonies ÷ *y* sample type); this generated the average number of *Burkholderia* recovered from these samples.

Through this simple analysis, it can be seen that plant roots appeared to generate a higher recovery rate when compared to the other sample types taken throughout this study. The recovery rate from leaf litter and rotting wood was particularly poor. This may be due to excess exposure to limiting factors such as desiccant properties of sunlight and poor nutrient availability.

Figure 3.4 Effects of seasonal conditions on viable *Burkholderia* recovery from the environment (number of confirmed *Burkholderia* isolates = 96)



The figures for the above graph were calculated from the number of samples taken during the various seasons and the number of confirmed *Burkholderia* isolates obtained from the samples during the different seasons.

The numbers of *Burkholderia* recovered per sample increased during the summer months. During the spring month the recovery rate was almost half of the summer months. However, during the winter months there were no sampling carried out therefore it was not clear whether summer was the peak time for *Burkholderia* sampling or whether there was another peak during winter.

3.7 Fatty acid profile / FAME analysis of novel *Burkholderia* isolates

Once the presumptive novel *Burkholderia* isolates were identified by the *Burkholderia*-specific PCR, fatty acid profile analysis commenced to get an initial speciation of the isolate. Out of the 96 novel isolates selected for FAP analysis, 82 presumptive *Burkholderia* cultures were able to grow *in vitro*. Fifty two presumptive *Burkholderia* isolates produced sufficient growth on the TSBA plates for fatty acid analysis (see appendix A6 for full names of the fatty acids) (31 profiles were not included in this table as problems were encountered with the printing of data sheets). The Richardson ID was based on a library of FAPs of fully characterised and environmental *Burkholderia* isolates collected by Jane Richardson as part of her PhD studies. The Microbial ID is based on wide collection of fully characterised bacteria from all genera.

Table 3.6 Fatty acid profile FAP / FAME analysis of 51 novel *Burkholderia* isolates and type strains

Out of the 82 Burkholderia isolated from the initial PCAT plates and the *Burkholderia* specific PCR analysis 51 presumptive *Burkholderia* isolates underwent fatty acid profiling. Not all of the 82 *Burkholderia* isolates are included, 26 did not produce sufficient growth for the fatty acid profile analysis and 5 after testing were not from the *Burkholderia* genus (results not shown).

Each fatty acid is a percentage of total fatty acid identified. The table only shows the key fatty acids found in *Burkholderia* species.

Isolate ID	10:0	12:0	13:1	15:1 w6c	14:0	14:0 3OH*	16:0	17:0 cycio	16:1 2OH	16:0 2OH	16:0 3OH	18:1 w7c	18:0	19:0 cyclo w8c	18:1 2OH	Microbial I.D	Richardson I.D
C5AIM	-	-	-	-	3.38	4.73	13.44	2.12	2.26	1.78	4.36	45.45	1.05	1.73	1.16	*B. cepacia*	*Burkholderia* sp cluster 11
C4DIM	-	-	-	-	3.43	5.16	13.31	1.61	2.32	1.90	4.21	45.66	-	1.71	1.29	*B. cepacia*	*Burkholderia* sp cluster 5
CWP-01	-	2.51	0.27	-	0.50	5.69	15.57	2.84	1.25	1.85	4.25	-	1.09	1.70	0.70	*B. cepacia*	*Burkholderia* sp cluster 6
CWP-02	-	2.86	-	-	-	5.59	13.80	2.27	2.06	2.41	4.86	-	-	1.39	-	*B. cepacia*	
CWP-03	-	4.91	-	-	-	4.91	17.23	2.29	1.71	1.66	4.40	-	-	1.39	-	*B. cepacia*	*Burkholderia* sp cluster 11
CWR-01	-	-	-	-	3.35	4.61	12.69	3.77	1.62	1.80	4.35	-	-	-	-	*B. cepacia*	*Burkholderia* sp cluster 5
CWR-02	-	2.59	-	-	0.81	5.57	18.03	3.90	1.09	1.24	4.53	42.64	1.06	2.10	-	*B. cepacia*	*Burkholderia* sp cluster 9
CWR-03	-	-	-	-	3.22	5.44	15.11	4.58	1.21	1.95	4.19	-	1.07	-	-	*B. cepacia*	*Burkholderia* sp cluster 16
CWR-04	0.05	2.41	1.03	0.15	0.58	5.55	16.28	3.54	1.31	1.09	3.04	40.60	0.74	1.96	0.53	*B. pyrrocinia*	
CWS-01	-	2.55	-	-	-	5.52	16.12	5.08	1.41	1.63	5.04	-	-	3.00	-	*B. cepacia*	*Burkholderia* sp cluster 6
CWS-02	-	-	-	-	3.24		14.85	-	1.06	1.24	4.52	-	1.06	2.10	-	*B. cepacia*	
HWP-12	-	1.40	0.36	-	2.03	4.92	16.30	2.66	1.21	1.68	3.97	43.67	1.03	2.00	0.74	*B. glathei*	*B. andropogonis*
HWP-18	-	2.66	0.54	0.12	0.48	4.82	20.55	3.14	1.26	1.60	3.76	36.88	0.71	1.83	0.32	*B. pyrrocinia*	*B. cepacia* cluster 13

Isolate ID	10:0	12:0	13:1	15:1 w6c	14:0	14:0 3OH*	16:0	17:0 cyclo	16:1 2OH	16:0 2OH	16:0 3OH	18:1 w7c	18:0	19:0 cyclo w8c	18:1 2OH	Microbial I.D	Richardson I.D
HWP-19	0.07	2.10	0.25	0.11	0.96	4.72	18.65	3.22	1.67	1.78	3.75	41.92	0.84	1.63	0.59	*B. cepacia*	*B. cepacia* cluster 13
HWP-20	0.10	2.57	0.38	0.16	0.49	4.76	21.41	3.35	1.21	1.41	3.74	36.25	0.67	2.03	-	*B. pyrrocinia*	*B. cepacia* cluster 13
HWP-21	-	2.06	0.36	0.09	0.94	4.67	18.85	2.75	1.55	1.60	3.66	42.25	0.89	1.36	0.56	*B. pyrrocinia*	*B. cepacia* cluster 13
HWP-22	0.10	2.52	0.30	0.12	0.50	4.82	16.28	3.93	1.44	2.22	4.01	42.24	0.79	1.96	0.53	*B. glathei*	*B. andropogonis*
HWR-10	0.08	2.48	0.40	-	0.68	4.60	14.27	3.87	1.24	1.97	3.90	45.87	0.49	3.06	0.90	*B. glathei*	*B. andropogonis*
HWR-15	-	1.28	0.41	-	1.96	4.50	14.68	3.36	1.85	2.02	3.35	44.35	0.93	2.16	1.27	*B. glathei*	*B. andropogonis*
HWR-20	-	1.25	0.38	-	1.83	4.36	16.84	2.13	1.53	2.00	3.21	45.12	1.14	1.40	1.25	*B. cepacia*	*B. cepacia* cluster 13
HWS-03	-	-	0.38	-	3.19	5.17	14.32	6.92	1.63	2.67	4.33	44.02	1.02	-	0.79	*B. glathei*	*B. caryophylli*
HWS-21	0.05	2.24	0.27	0.11	0.84	4.85	19.37	4.37	2.04	2.30	3.96	39.23	0.66	2.05	0.78	*B. cepacia*	*B. cepacia* cluster 13
HWS-25	0.08	2.38	0.35	0.11	0.73	4.85	14.42	5.27	0.92	1.56	3.80	42.18	0.84	2.54	0.54	*B. glathei*	*B. caryophylli* cluster 13
HWS-29	0.09	2.52	0.20	0.15		5.21	16.74	5.93	3.00	3.00	4.10	35.14	0.38	2.76	0.97	*B. cepacia*	*B. gladioli*
HWS-32	0.10	2.37	0.34	0.16	0.87	5.61	15.38	3.90	1.68	2.02	4.35	39.87	0.53	1.62	0.68	*B. pyrrocinia*	*B. andropogonis*
HWS-34	0.06	2.06	0.23	0.17	0.78	4.73	16.96	2.58	1.49	1.87	3.65	42.80	0.79	1.01	0.52	*B. pyrrocinia*	*B. andropogonis*
HWS-35	0.08	2.06	0.49	-	0.85	4.79	19.07	1.80	1.13	1.40	3.53	42.64	1.11	0.80	0.38	*B. pyrrocinia*	*B. cepacia* cluster 13
HWS-36	-	-	-	-	3.59	5.09	16.24	4.13	0.99	1.01	4.03	46.38	0.66	1.25	0.50	*B. glathei*	*B. caryophylli*
HWS-44	-	-	-	-	4.25	3.93	25.19	8.34	0.70	0.86	3.04	25.59	1.13	3.04	0.27	*B. cepacia*	*B. gladioli* cluster 1
HWS-45	0.05	2.14	0.36	0.13	1.12	4.74	19.13	0.18	1.44	1.43	3.63	41.41	0.76	1.09	0.51	*B. pyrrocinia*	*B. cepacia* cluster 13
HWS-46	-	1.80	0.35	0.12	1.33	4.79	15.33	1.80	1.73	2.19	3.88	48.94	0.47	0.83	0.88	*B. glathei*	*B. cepacia* cluster 13
HWS-47	0.09	2.10	0.38	-	0.90	4.85	17.84	1.91	1.00	1.43	3.74	46.64	1.23	1.41	0.57	*B. cepacia*	*B. cepacia* cluster 13
HWS-48	0.10	2.11	0.40	0.08	0.95	4.71	18.08	1.84	1.00	1.35	3.62	46.07	1.13	1.28	0.54	*B. cepacia*	*B. cepacia* cluster 13
HWS-49	-	2.12	0.36	-	1.04	4.87	18.12	1.74	1.17	1.33	3.82	45.07	1.03	1.00	0.52	*B. cepacia*	*B. cepacia*

Isolate ID	10:0	12:0	13:1	15:1 w6c	14:0	14:0 3OH*	16:0	17:0 cycio	16:1 2OH	16:0 2OH	16:0 3OH	18:1 w7c	18:0	19:0 cyclo w8c	18:1 2OH	Microbial I.D	Richardson I.D
KPS-02	-	1.32	0.28	-	1.93	5.08	16.91	1.34	1.44	1.61	4.18	41.46	1.44	0.83	0.58	*B. pyrrocinia*	cluster 13 *B. cepacia*
KPS-07	-	-	0.48	-	3.68	4.77	18.64	5.94	0.30	1.41	4.31	42.54	0.56	1.48	0.37	*B. glathei*	cluster 2 *B. caryophylli*
P13B	-	-	0.80	-	3.34	4.21	23.30	9.13	0.64	0.69	2.81	33.26	1.52	3.58	0.27	*B. glathei*	*B. gladioli*
P13F	-	-	0.33	-	3.38	4.25	22.86	9.68	0.80	1.01	3.21	29.10	1.38	4.19	0.32	*B. cepacia*	*B. gladioli*
YWS-01	0.09	2.24	0.4	0.10	0.82	5.17	19.59	2.69	1.72	2.20	4.21	41.27	0.76	1.59	0.70	*B. cepacia*	*B. caryophylli*
YWS-02	-	2.10	0.47	-	0.89	4.98	17.92	1.14	1.44	1.61	4.07	44.04	1.02	0.74	0.60	*B. pyrrocinia*	*B. cepacia*
YWS-03	-	2.36	0.56	-	0.83	5.58	16.04	6.13	2.35	2.33	4.57	40.14	0.54	2.92	0.97	*B. glathei*	cluster 13 *B. caryophylli*
YWS-05	-	2.17	0.46	-	0.91	5.30	17.00	2.18	1.66	1.46	4.10	43.52	1.27	1.37	0.61	*B. pyrrocinia*	*B. cepacia*
YWS-06	-	1.07	0.67	-	2.75	5.60	15.96	8.73	1.73	2.49	4.62	34.48	0.70	5.10	0.85	*B. glathei*	cluster 13 *B. gladioli*
YWS-07	-	1.05	0.36	0.39	2.81	5.59	17.61	12.65	2.02	2.95	4.75	27.70	0.48	8.36	0.88	*B. gladioli*	*B. gladioli*
YWS-12	-	2.61	.65	-	1.27	5.47	16.84	8.05	2.02	2.21	4.91	35.02	-	2.77	1.01	*B. glathei*	*B. gladioli*
YWS-14	-	1.17	0.71	-	3.15	5.93	16.20	11.79	2.21	3.04	5.00	30.18	0.67	7.45	1.02	*B. glathei*	*B. gladioli*
YWS-15	-	1.06	0.45	0.21	3.00	5.81	16.76	14.64	1.75	2.99	5.06	25.68	0.48	11.25	0.81	*B. gladioli*	*B. gladioli*
B. amb[1]	-	-	0.31	-	3.92	5.56	14.97	6.00	1.66	2.27	2.58	44.29	0.86	4.31	1.59	*B. cepacia*	
B. cal[2]	-	0.87	0.59	-	2.72	4.54	18.38	7.81	0.61	0.82	2.78	38.13	0.85	5.38	2.02	*B .cepacia*	*B. gladioli*
B. fun[3]	-	0.89	0.51	-	2.80	4.45	17.79	8.79	0.58	0.79	2.86	36.52	0.78	6.49	1.96	*B. cepacia*	*B. glumae*
B. sac[4]	-	0.05	0.48	0.06	2.63	5.36	19.08	6.68	0.95	1.18	3.01	41.05	0.80	1.22	0.80	*B. glathei*	

- ***sum of several fatty acids within a feature containing 16:1 ISO I.***
- *** In the nomeclature of fatty acids the first number indicates the length of the carbon chain, the second number represents double bonds and the final part indicates the presence of functional groups such as hydroxyl groups (OH) attached to the carbon chain. *ie* 14:0 3OH is 14 carbon atoms long, no double bonds and has a hydroxyl group attached to the third carbon atom.**

- [1] *B. ambifaria* LMG 19182, [2] *B. caledonica* LMG 19076, [3] *B. fungorum* LMG 16225, [4] *B. sacchari* LMG 19450

3.8 Fatty acid profile analysis of novel isolates and *Burkholderia* type strains

Out of 70 isolates tested, 45 were tentatively identified as *Burkholderia* species according to the Microbial I.D system. The primary 5 hydroxyl acids found in *Burkholderia* species are 14:0 3OH, 16:1 2OH, 16:0 2OH, 16:0 3OH and 18:1 2OH. The FAPs of the novel isolates were compared to type strains of various *Burkholderia* species.

3.9 Comparative study of fatty acid profiles at time 24 and 48 hours

Due to poor growth, some isolates required more than 5 TSBA plates from a 24 hour incubation to harvest enough cells for FAP analysis. The effects of an extended incubation period on the FAPs generated by the *Burkholderia* isolates were investigated. The outcome of this was from the extended incubation period, the isolates produced different FAPs (table 3.7). One particular fatty acid, 19:0 cyclo ω8c, appeared after 24 hours. In CWP-01 at 24 hours there was 19:0 cyclo ω8c present (1.7%), but the level increased vastly after 48 hours incubation (3.17%).

Table 3.7 Effects of an extended incubation time on the FAP ID

The table below shows the difference of the fatty acid 19:0 cyclo levels after 24 hours and 48 hours.

Isolate	FAP ID after 24 h	19:0 cyclo (%)	FAP ID after 48 h	19:0 cyclo (%)
CWS-01	*B. cepacia* cluster 16	0%	*B. cepacia* cluster 11	0.44%
CWP-01	*B. cepacia* cluster 6	1.7%	*B. cepacia* cluster 6	3.17%
CWR-03	*B. cepacia* cluster 16	0%	*B. cepacia* cluster 8	0.47%

The effects of an extended incubation period revealed that the levels of the fatty acid, 19:0 cyclo increased. The change in proportion of the fatty acids present often changed the FAP ID and cluster group of the isolate tested.

Table 3.8 Colony characterisation and sources of 82 novel environmental isolates

All novel environmental isolates had the *Burkholderia*-like colonial morphology which can be described as shiny, dome shaped and cream in colour. However, the occasional novel isolate had some pigmentation similar to the *B. cepacia* type strain 2993. The table consists of the isolates confirmed by the *Burkholderia*-specific PCR analysis.

ID Number	Organism	Isolation media *	Background
CWR-01	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) rotting wood, woodlands, Sand Hutton, York, UK.
CWR-02	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) rotting wood, woodlands, Sand Hutton, York, UK.
CWR-04	*Burkholderia sp.*	PCAT	*Laurus* sp. (laurel) – root, woodlands, Sand Hutton York, UK.
CWR-06	*Burkholderia sp.*	PCAT	*Fraxinus* sp.Ash- rotting wood, woodlands, Sand Hutton, York, UK.
CWS-01	*Burkholderia sp.*	PCAT	*Quercus* sp (oak) –rhizospheric soil, woodlands, Sand Hutton, York, UK.
CWS-02	*Burkholderia sp*	PCAT	*Quercus* sp (oak) - rhizospheric soil, woodlands, Sand Hutton, York, UK.
CWS-03	*Burkholderia sp.*	PCAT	*Platanus* sp. (sycamore) rhizospheric soil, woodlands, Sand Hutton, York, UK.
CWP-01	*Burkholderia sp.*	PCAT	*Laurus* sp. (laurel) -root, woodlands, Sand Hutton, York, UK.
CWP-02	*Burkholderia sp.*	PCAT	*Laurus* sp. (laurel) -root, woodlands, Sand Hutton, York, UK.
CWP-03	*Burkholderia sp.*	PCAT	*Laurus* sp. (laurel) root , woodlands, Sand Hutton, York, UK.
CWL-01	*Burkholderia sp.*	PCAT	*Laurus* sp. (laurel) Leaf litter, woodlands, Sand Hutton, York, UK.
C3AIM	*B. graminis*		*Graminacae* sp. -Maize root macerate, Lyon, France. Donated by Dr J. Balandreau.
C4DIM	*B. graminis*		*Graminacae* sp. -Maize root macerate, Lyon, France. Donated by Dr J. Balandreau.
C5AIM	*B. graminis*		*Graminacae* sp. -Maize root macerate, Lyon, France. Donated by Dr J. Balandreau.
CUP	*Burkholderia sp.*	SMSA[1]	Plant sample- root. Donated by Simon Weller, CSL, York, UK.
HWL-11	*Burkholderia sp.*	PCAT	*Betula* sp. (birch) leaf litter, woodlands, Hampstead Heath, London UK.
HWL-11	*Burkholderia sp.*	PCAT	*Betula* sp. (birch) leaf litter, woodlands, Hampstead Heath, London, UK.
HWP-10	*Burkholderia sp.*	PCAT	*Fagus* sp. (beech) - moss, woodlands, Hampstead Heath, London, UK.
HWP-11	*Burkholderia sp.*	PCAT	*Fagus* sp. (beech)- moss, woodlands, Hampstead Heath, London, UK.
HWP-12	*Burkholderia sp.*	PCAT	*Fagus* sp. (beech)- moss, woodlands, Hampstead Heath, London, UK.
HWP-13	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) - moss, woodlands, Hampstead Heath, London, UK.
HWP-14	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak)- moss, woodlands, Hampstead Heath, London, UK.
HWP-15	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak)- moss, woodlands, Hampstead Heath, London, UK.
HWP-16	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak)- moss, woodlands, Hampstead Heath, London, UK.
HWP-20	*Burkholderia sp.*	PCAT	*Fagus* sp. (beech)- moss, woodlands, Hampstead Heath, London, UK.
HWP-21	*Burkholderia sp.*	PCAT	*Fagus* sp. (beech)- moss, woodlands, Hampstead Heath, London, UK.
HWR-10	*Burkholderia sp.*	PCAT	Unknown plant root, woodlands, Hampstead Heath, London, UK.
HWS-01	*Burkholderia sp.*	PCAT	*Salix* sp. rhizospheric soil, woodlands, Hampstead Heath, London, UK
HWS-02	*Burkholderia sp.*	PCAT	*Betula pendula* (silver birch) rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-03	*Burkholderia sp.*	PCAT	*Fagus* sp. (beech) rhizospheric soil, woodlands, Hampstead Heath, London, UK.

ID Number	Organism	Isolation media *	Background
HWS-04	*Burkholderia sp.*	PCAT	*Fagus* sp. (beech) rhizospheric soil woodlands, Hampstead Heath, London, UK.
HWS-05	*Burkholderia sp.*	PCAT	*Pinus* sp. (pine) rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-20	*Burkholderia sp.*	PCAT	*Betula* sp. (birch) –rhizospheric soil, Hampstead Heath, London, UK.
HWS-21	*Burkholderia sp.*	PCAT	*Betula* sp. (birch) –rhizospheric soil, Hampstead Heath, London, UK.
HWS-22	*Burkholderia sp.*	PCAT	*Betula* sp. (birch) –rhizospheric soil, Hampstead Heath, London, UK.
HWS-23	*Burkholderia sp.*	PCAT	*Fraxinus* sp. (ash) –rhizospheric soil, Hampstead Heath, London, UK.
HWS-24	*Burkholderia sp.*	PCAT	*Betula* sp.(birch) –rhizospheric soil, Hampstead Heath, London, UK.
HWS-25	*Burkholderia sp.*	PCAT	*Betula* sp. (birch) –rhizospheric soil, Hampstead Heath, London, UK.
HWS-26	*Burkholderia sp.*	PCAT	*Fagus* sp. (beech) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-27	*Burkholderia sp.*	PCAT	*Fagus* sp. (beech) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-28	*Burkholderia sp.*	PCAT	*Fagus* sp. (beech) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-29	*Burkholderia sp.*	PCAT	*Fagus* sp. (beech) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-30	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-31	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-32	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-33	*Burkholderia sp.*	PCAT	*Platanus* sp. (sycamore)- rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-34	*Burkholderia sp.*	PCAT	*Platanus* sp. (sycamore) – rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-35	*Burkholderia sp.*	PCAT	*Platanus* sp. (sycamore) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-36	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-37	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-38	*Burkholderia sp.*	PCAT	*Fagus* sp. (beech) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-39	*Burkholderia sp.*	PCAT	*Fagus* sp. (beech) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-40	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-41	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-42	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-43	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
HWS-44	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
KPS-02	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak)- rhizospheric soil, open gardens, Kensington Gardens, London, UK.
KPS-07	*Burkholderia sp.*	PCAT	*Fagus* sp. (beech) –rhizospheric soil, open gardens, Kensington Gardens, London, UK.
P13B	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
P13F	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) –rhizospheric soil, woodlands, Hampstead Heath, London, UK.
YWP-18	*Burkholderia sp.*	PCAT	*Vaccinium myrtillus L.* (bilberry plant) root, roadside bank, Sand Hutton, York, UK.
YWP-19	*Burkholderia sp.*	PCAT	*Vaccinium myrtillus L* (bilberry plant) root, roadside bank, Sand Hutton, York, UK.
YWP-20	*Burkholderia sp.*	PCAT	*Vaccinium myrtillus* L (bilberry plant) root, roadside bank, Sand Hutton, York, UK.
YWP-21	*Burkholderia sp.*	PCAT	*Vaccinium myrtillus L* (bilberry plant) root, roadside bank, Sand Hutton, York, UK.
YWP-22	*Burkholderia sp.*	PCAT	*Vaccinium myrtillus L* (bilberry plant) root, roadside bank, Sand Hutton, York, UK.

ID Number	Organism	Isolation media *	Background
YWS-01	*Burkholderia sp.*	PCAT	*Fagus* sp. (beech) rhizospheric soil, woodlands, Sand Hutton, York, UK.
YWS-02	*Burkholderia sp.*	PCAT	*Ulmus* sp. (elm) rhizospheric soil, woodlands, Sand Hutton, York, UK.
YWS-03	*Burkholderia sp.*	PCAT	*Ulmus* sp. (elm) rhizospheric soil, woodlands, Sand Hutton, York, UK.
YWS-04	*Burkholderia sp.*	PCAT	*Ulmus* sp. (elm) rhizospheric soil, woodlands, Sand Hutton, York, UK.
YWS-05	*Burkholderia sp.*	PCAT	*Fraxinus* sp. (ash) rhizospheric soil, woodlands, Sand Hutton, York, UK.
YWS-06	*Burkholderia sp.*	PCAT	*Betula pendula* (silver birch) rhizospheric soil, woodlands, Sand Hutton, York, UK.
YWS-07	*Burkholderia sp.*	PCAT	*Betula pendula* (silver birch) rhizospheric soil, woodlands, Sand Hutton, York, UK.
YWS-12	*Burkholderia sp.*	PCAT	*Betula pendula* (silver birch) rhizospheric soil, woodlands, Sand Hutton, York, UK.
YWS-13	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) rhizospheric soil, woodlands, Sand Hutton, York, UK.
YWS-14	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) rhizospheric soil, woodlands, Sand Hutton, York, UK.
YWS-15	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) rhizospheric soil, woodlands, Sand Hutton, York, UK.
YWS-45	*Burkholderia sp.*	PCAT	*Quercus* sp. (oak) rhizospheric soil, woodlands, Sand Hutton, York, UK.
YWS-46	*Burkholderia sp.*	PCAT	*Acer pseudoplatanus* (sycamore) –rhizospheric soil, Sand Hutton, York, UK.
YWS-47	*Burkholderia sp.*	PCAT	*Betula pendula* (silver birch)- rhizospheric soil, Sand Hutton, York, UK.
YWS-48	*Burkholderia sp.*	PCAT	*Betula pendula* (silver birch)- rhizospheric soil, Sand Hutton, York, UK.
YWS-49	*Burkholderia sp.*	PCAT	*Betula pendula* (silver birch)- rhizospheric soil, Sand Hutton, York, UK.

*** when not allocated media name, it is not known which media was used in the initial isolation**

[1]SMSA – Modified semi-selective media (Engelbretcht, 1994)

3.10 Growth of novel environmental isolates at 28°C and 37°C

Using growth at 37°C as an indicator of potential human pathogenicity, the novel environmental isolates were incubated both at 28°C and 37°C for 24-48h (see table 3.9 for results). Not all of the isolates confirmed as *Burkholderia* were selected for this experiment. The isolates were selected according to how they grouped with the human pathogenic strains in the BOX and ERIC DNA fingerprinting dendrograms. The BOX and ERIC DNA dendrograms are contained in the next chapter as part of the genotypic characterisation of the novel isolates.

Table 3.9 Growth of novel environmental isolates at 28°C and 37°C

The amounts of growth with each isolate were observed visually after 24h and 48h at each temperature. The growth from the isolates after 48h was recorded on the table below. The ability to grow at the two temperatures was compared. *B. cepacia* 2993 type strain (plant pathogenic) and *B. cepacia* NCPPB 3025 (environmental – onion pathogenic) were used as the 28°C growth control and the *B. cepacia* J2315 (human pathogenic) was used as the 37°C growth control. The experiment provided a 'snap-shot' review of environmental isolates ability to adapt to growth at higher temperatures.

Bacterial ID	Growth at 28°C	Growth at 37°C	Origins
***B. cepacia* strains**			
J2315	+++	+++	Clinical- CF patient -epidemic strain
NCPPB 3025	+++	+++	Environmental -Onion pathogenic
2993	+++	+++	Onion- Type strain
Novel environmental *Burkholderia* isolates			
CWL-01	+++	+++	Environmental
CUP	+++	+++	Environmental
CWP-01	+++	+ w	Environmental
CWP-02	+++	+++	Environmental
CWS-01	+++	+++	Environmental
CWS-02	+++	+++	Environmental
CWS-03	+++	+++	Environmental
HWS-03	+++	-	Environmental
HWS-04	+++	-	Environmental
HWS-21	+++	-	Environmental
KPS-02	+++	-	Environmental
YWS-01	+++	-	Environmental
YWS-05	+++	+w	Environmental
YWS-06	+++	-	Environmental
YWS-07	+++	-	Environmental
YWS-13	+++	-	Environmental

Key: **+++** strong growth **++** moderate growth **+w** weak growth
- no growth

A selection of 16 novel isolates were chosen to investigate the ability to grow at 37°C. The selection on these isolates were based on the sample origin, representative isolates were taken to cover the majority of samples taken from the woodland sites. Out of the novel isolates 8 showed signs of growth at 37°C, 6 grew well at this temperature. CWL-01, CUP, CWP-02, CWS-01 CWS-02 and CWS-03 all had a good ability to grow at 37°C, therefore illustrating their potential for human colonisation as to cause infection.

3.11 Onion pathogenicity test of novel *Burkholderia* isolates

The 39 novel *Burkholderia* isolates were selected for this particular experiment because they grouped closely to known onion pathogenic strains of *Burkholderia* species on the 16S rDNA phylogram (see chapter 4).

Table 3.10 Onion pathogenicity test of novel *Burkholderia* isolates and other *Burkholderia* species.

The onion scales were checked periodically at 24h, 48h and 72h during incubated at 30°C. Each of the dish containing onion tissue was checked for any signs of onion pathogenicity after 72h of incubation.

Isolate name	Ability to rot onion (–ve or +ve)
CUP	-ve
CWL-11	-ve
CWP-01	-ve
CWP-02	-ve
CWR-02	-ve
CWS-02	-ve
CWS-03	+ve sour smell, slimy and shiny appearance of onion tissue
HWP-12	-ve
HWP-13	-ve
HWP-15	-ve
HWP-16	-ve
HWL-11	-ve
HWS-03	-ve
HWS-04	-ve
HWS-20	-ve
HWS-21	-ve
HWS-22	-ve
HWS-28	-ve
HWS-29	-ve
HWS-30	-ve
HWS-31	-ve
HWS-33	-ve
HWS-34	-ve
HWS-36	-ve
HWS-37	-ve
HWS-39	-ve
HWS-41	-ve
HWS-43	-ve
HWS-44	-ve
KPS-02	-ve
KPS-07	-ve
YFS-01	-ve
YWS-01	-ve
YWS-02	-ve
YWS-04	-ve

Isolate name	Ability to rot onion (–ve or +ve)
YWS-05	-ve
YWS-07	-ve
YWS-13	-ve
YWS-15	-ve
Clinical *B. cepacia* samples	
10744[b]	-ve
13007[c]	-ve
13009[d]	-ve
13010[e]	-ve
13011[f]	-ve
Burkholderia graminis	
C3AIM	-ve
C4DIM	-ve
C5AIM	-ve
***Burkholderia* control strains for pathogenicity**	
J2315[a]	-ve
2993*	+ve Sour smell, slimy and shiny appearance of onion tissue.

*** Positive strain control- *B. cepacia* type strain, first isolate from an onion displaying symptoms of sour rot. [a] Negative strain control- *B. cepacia* clinical strain Edinburgh-Toronto epidemic strain, not known to cause maceration of onion tissues. [b] *B. cepacia* clinical strain from a CF patient. [c] *B. cepacia* clinical strain from a CF patient. [d] *B. cepacia* clinical strain from a CF patient. [e] *B. cepacia* clinical strain from a CF patient. [f] *B. cepacia* clinical strain from a CF patient- epidemic strain.**

Out of the novel environmental *Burkholderia* isolates tested, only CWS-03 produced the signs of onion pathogenicity through the maceration of the onion tissue. The colonial morphology of CWS-03 also demonstrated that it produced a yellow / green pigmentation similar to that of the pigment produced by *B. cepacia* type strain 2993.

3.12 Carbohydrate utilisation- API 20NE and API 50CH

The novel environmental isolates were selected according to how they clustered in the BOX and ERIC DNA fingerprint dendrograms (see chapter 4). Representative environmental isolates were taken from each group and characterised using the API 20NE (24 environmental isolates selected) and API 50CH (21 environmental isolates selected) strips. Not all novel isolates were tested for carbohydrate utilisation; a selection based on origin of sample was made to gain a 'snap-shot' of how the carbohydrate profiles varied between the woodland *Burkholderia* isolates. After incubation the colour change is recorded and referred to the manufacturers'

instructions to indicate whether the conventional or carbohydrate assimilation tests proved positive (see tables 3.11 and 3.12 for API20 NE and API 50CH results).

Table 3.11 API 20NE carbohydrate profile of novel *Burkholderia* isolates.

This assay comprises of 10 conventional tests and 12 assimilation tests for the identification of non fastidious negative rods not belonging to the group *Enterobacteriacae*. (+ = positive colour change, - = negative colour change)

	C1576 (Clinical)	C4DIM (Graminacae)	CWP-03 (Laurus)	CWR-06 (Fraxinus)	CWS-02 (Quercus)	CWS-03 (Platanus)	HWP-20 (Fagus)	HWS-03 (Fagus)	HWS-21 (Betula)	HWS-29 (Fagus)	HWS-37 (Quercus)	HWS-44 (Quercus)
NO2	+	-	-	-	-	-	-	-	-	+	-	-
N2	-	-	-	-	-	-	-	-	-	-	-	-
TRP	-	-	-	-	-	-	-	-	-	-	-	-
GLU	-	-	-	-	-	-	-	-	-	-	-	-
ADH	-	-	-	-	-	-	-	-	-	-	-	-
URE	-	-	-	+	-	+	-	-	-	-	-	-
ESC	-	-	-	-	-	-	-	-	+	+	-	-
GEL	-	-	-	-	-	-	-	-	-	-	-	-
PNG	-	-	+	+	-	+	+	-	+	+	-	+
GLU	+	+	+	+	+	+	+	+	+	+	+	+
ARA	+	-	+	-	+	-	+	+	-	-	+	+
MNE	+	-	+	-	+	-	+	+	-	+	+	+
MAN	+	-	+	+	+	+	+	+	+	+	+	+
NAG	+	+	+	+	+	+	+	+	+	+	+	+
MAL	-	+	-	-	-	-	-	-	-	-	-	+
GNT	+	-	+	+	+	+	+	+	+	+	+	+
CAP	+	-	+	-	-	-	+	+	-	+	+	+
ADI	+	-	+	+	+	+	+	+	+	+	+	+
MLT	+	-	+	+	+	+	+	+	+	+	+	+
CIT	+	-	+	+	-	+	-	+	-	-	+	+
PAC	+	-	+	+	+	+	+	+	+	+	+	+
OX	-	-	+	+	-	-	-	+	+	+	+	+

API 20NE continued

	OY715 (Allium cepa)	P13F (Quercus)	YWP-18 (Vaccinium myrtillus L)	YWP-19 (Vaccinium myrtillus L.)	YWP-20 (Vaccinium myrtillus L)	YWP-21 (Vaccinium mrytillus L)	YWP-22 (Vaccinium mrytillus L)	YWS-45 (Quercus)	YWS-46 (Acer)	YWS-47 (Betula)	YWS-48 (Betula)	YWS-49 (Betula)
NO2	-	-	-	-	-	-	-	-	-	-	-	-
N2	-	-	-	-	-	-	-	-	-	-	-	-
TRP	-	-	-	-	-	-	-	-	-	-	-	-
GLU	-	-	-	-	-	-	-	-	-	-	-	-
ADH	-	-	-	-	-	-	-	-	-	-	-	-
URE	-	-	-	-	-	-	-	-	-	-	-	-
ESC	-	+	-	-	-	-	-	-	-	-	-	-
GEL	-	-	-	-	-	-	-	-	-	-	-	-
PNG	+	+	-	-	-	-	-	-	-	-	-	-
GLU	+	+	+	+	+	+	+	+	+	+	+	+
ARA	+	-	+	+	+	+	+	+	+	+	+	+
MNE	+	+	+	+	+	+	+	+	+	+	+	+
MAN	+	+	+	+	+	+	+	+	+	+	+	-
NAG	+	+	+	+	+	+	+	+	+	+	+	+
MAL	-	-	-	-	+	-	-	-	-	-	-	-
GNT	+	+	+	+	-	+	+	+	+	+	+	+
CAP	+	+	+	+	+	-	+	+	+	+	+	+
ADI	-	+	+	+	+	+	+	+	+	+	+	+
MLT	+	+	+	+	+	+	+	+	+	+	+	+
CIT	-	+	+	+	+	+	+	+	+	+	+	+
PAC	+	+	+	+	+	+	+	+	+	+	+	+
OX	+	+	+	+	-	-	-	-	+	-	-	-

Table 3.12 API 50CH carbohydrate profile of novel *Burkholderia* isolates (see appendix A6 for full name of carbohydrates)

This assay tests for the fermentation of 49 different carbohydrates. During incubation the carbohydrates are utilised and acidic by products formed which decreases the pH resulting in a colour change. This test was chosen because the carbohydrate utilisation profile can been used in complement with the API 20NE.

	2993 (Allium cepa)	C1576 (clinical)	C4DIM (Graminacae)	CWP-01 (Laurus)	CWR-01 (Quercus)	CWR-06 (Fraxinus)	CWS-02 (Quercus)	CWS-03 (Platanus)	HWS-03 (Fagus)	HWS-29 (Fagus)	J2315 (clinical)	KPS-02 (Quercus)	KPS-07 (Fagus)	OY715 (Allium cepa)
GLY	-	-	-	+	+	-	+	-	-	+	-	-	-	+
ERY	-	-	-	-	+	-	+	-	-	-	-	-	-	+
D-ARA	-	-	-	-	+	-	+	-	-	+	-	-	-	+
L-ARA	+	+	-	-	+	-	+	-	-	+	-	-	-	+
RIB	-	+	-	-	+	-	+	-	-	+	-	-	-	+
D-XYL	-	+	-	-	+	-	+	-	-	+	-	-	-	+
L-XYL	-	+	-	-	+	-	+	-	-	+	-	-	-	+
ADO	-	+	-	+	+	-	+	-	-	+	-	+	-	+
β-MDX	-	-	-	-	+	-	+	-	-	+	-	-	-	+
GAL	+	+	+	+	+	-	+	-	+	+	+	+	-	+
GLU	+	+	+	+	+	-	+	-	+	+	+	+	-	+
FRU	-	+	+	+	+	-	+	-	-	+	-	+	-	+
MNE	-	+	+	+	+	-	+	-	-	+	-	+	-	+
SBE	-	-	+	-	+	-	-	-	-	+	-	-	-	+
RHA	-	-	+	-	+	-	+	-	-	+	-	+	-	+
DUL	-	+	+	+	+	-	+	-	-	+	-	-	-	+
INO	+	+	-	+	+	-	+	-	-	+	-	+	-	+
MAN	+	+	+	+	+	-	+	-	-	+	-	+	-	+
SOR	+	+	-	+	+	-	+	-	-	+	-	+	-	+
α-MDM	-	-	-	-	-	-	-	-	-	-	-	-	-	-

API 50CH continued.

	2993 (Allium cepa)	C1576 (clinical)	C4DIM (Graminacae)	CWP-01 (Laurus)	CWR-01 (Quercus)	CWR-06 (Fraxinus)	CWS-02 (Quercus)	CWS-03 (Platanus)	HWS-03 (Fagus)	HWS-29 (Fagus)	J2315 (clinical)	KPS-02 (Quercus)	KPS-07 (Fagus)	OY715 (Allium cepa)
α-MDG	-	-	-	-	+	-	-	-	-	-	-	-	-	-
NAG	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AMY	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ARB	+	-	-	-	-	-	-	-	-	-	-	-	-	-
ESC	+	-	-	-	+	-	-	-	-	+	-	-	-	-
SAL	+	-	-	-	+	-	-	-	-	+	-	-	-	-
CELI	+	+	-	-	+	-	-	-	-	+	-	-	-	-
MAL	+	+	-	-	+	-	-	-	-	+	-	-	-	-
LAC	+	+	-	-	+	-	-	-	-	+	-	-	-	-
MEL	-	-	-	-	+	-	-	-	-	-	-	-	-	-
SAC	+	-	+	-	+	-	-	-	-	-	-	-	-	-
TRE	-	+	-	-	+	-	-	-	-	-	-	-	-	-
INU	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MLZ	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RAF	-	-	-	-	+	-	-	-	-	-	-	-	-	-
STA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GLYG	-	-	-	-	-	-	-	-	-	-	-	-	-	-
XLT	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GEN	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DTUR	-	-	-	-	+	-	-	-	-	-	-	-	-	-
DLYX	-	-	-	-	+	-	-	-	-	-	-	-	-	-
DTAG	-	-	-	-	+	-	-	-	-	+	-	-	-	-
DFUC	+	+	-	-	+	-	-	-	-	+	-	-	-	-
LFUC	-	+	-	+	+	+	+	-	-	+	-	-	-	+
DARL	-	+	-	+	+	-	+	-	-	+	-	-	-	+
LARL	-	+	-	+	+	-	+	-	-	+	-	-	-	-
GNT	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2KG	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5KG	-	-	-	-	-	-	-	-	-	-	-	-	-	-

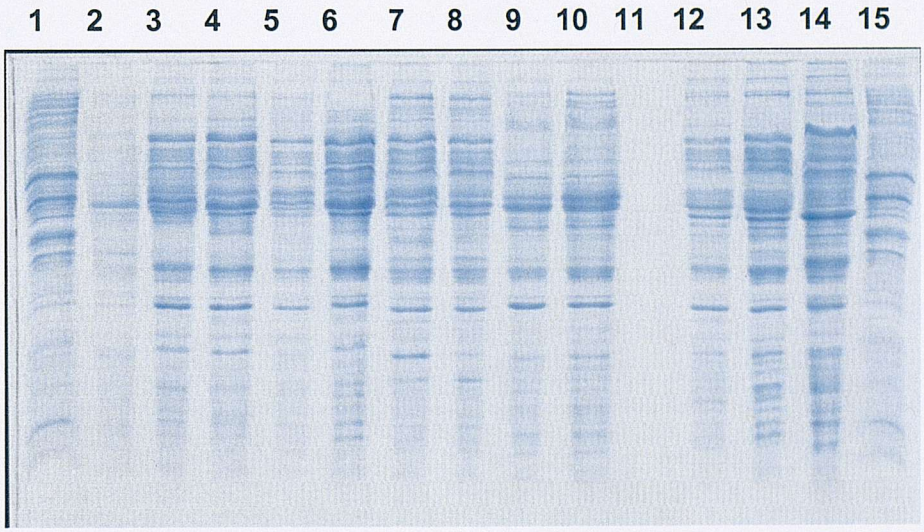
	YWP-18	YWP-20	YWP-22	YWP-45	YWP-47	YWP-48	YWP-49
GLY	-	-	+	+	+	+	+
ERY	-	-	-	-	-	-	-
D-ARA	-	-	-	+	+	+	+
L-ARA	-	-	-	-	+	+	+
RIB	-	-	-	+	+	+	+
D-XYL	-	-	-	+	+	-	+
L-XYL	-	-	-	-	-	-	+
ADO	-	-	+	-	+	+	+
β-MDX	-	-	-	+	-	-	+
GAL	-	-	+	-	+	+	+
GLU	-	-	+	+	+	+	+
FRU	-	-	+	+	+	+	+
MNE	-	-	+	+	+	+	+
SBE	-	-	-	-	-	-	-
RHA	-	-	+	+	+	+	+
DUL	-	-	-	-	-	-	+
INO	-	-	+	-	+	+	+
MAN	-	-	+	+	+	+	+
SOR	-	-	+	+	+	+	+
α-MDM	-	-	-	+	-	-	-
α-MDG	-	-	-	-	-	-	-
NAG	-	-	-	-	-	-	-
AMY	-	-	-	-	-	-	-
ARB	-	-	-	-	-	-	-
ESC	-	-	+	-	-	-	-
SAL	-	-	-	-	-	-	-
CELI	-	-	-	-	-	-	-
MAL	-	-	-	-	-	-	-
LAC	-	-	-	-	-	-	-
MEL	-	-	-	-	-	-	-
SAC	-	-	-	-	-	-	-
TRE	-	-	-	-	-	-	-
INU	-	-	-	-	-	-	-
MLZ	-	-	-	-	-	-	-
RAF	-	-	-	-	-	-	-
STA	-	-	-	-	-	-	-
GLYG	-	-	-	-	-	-	-
XLT	-	-	-	-	-	-	-
GEN	-	-	-	-	-	-	-
DTUR	-	-	-	-	-	-	-
DLYX	-	-	-	-	-	-	-
DTAG	-	-	-	-	-	-	-
DFUC	-	-	-	-	-	-	-
LFUC	-	-	+	+	-	-	-
DARL	-	-	+	+	-	-	-
LARL	-	-	+	-	-	-	-
GNT	-	-	-	-	-	-	-
2KG	-	-	-	-	-	-	-
5KG	-	-	-	-	-	-	-

3.13 Protein profiling of novel isolates and *Burkholderia* species using SDS PAGE gel electrophoresis

The novel *Burkholderia* isolates used in this experiment were selected based on the BOX and ERIC fingerprinting dendrograms (see chapter 4). Each of the selected isolates were representative of a cluster contained within the dendrograms. The whole cell protein profiles of the *Burkholderia* isolates were compared visually and through the use of GelCompar (dendrogram not seen). However, the GelCompar program failed to successfully group the novel isolate profiles due to between gel differences of the marker protein profiles, this problem could not be resolved. Using visual comparison, differences in the protein profiles could be seen, but no similarities could be found due to genomovars, plant species or habitat specificity (see figures 3.5, 3.6, 3.7 and 3.8). *Psychrobacter immobilis* was chosen as a marker as it generated bands that allowed the alignment of the protein profiles of the *Burkholderia* isolates.

Figure 3.5 Protein profiling of *Burkholderia* using SDS PAGE gel electrophoresis: Gel 1 Novel environmental *Burkholderia* isolates

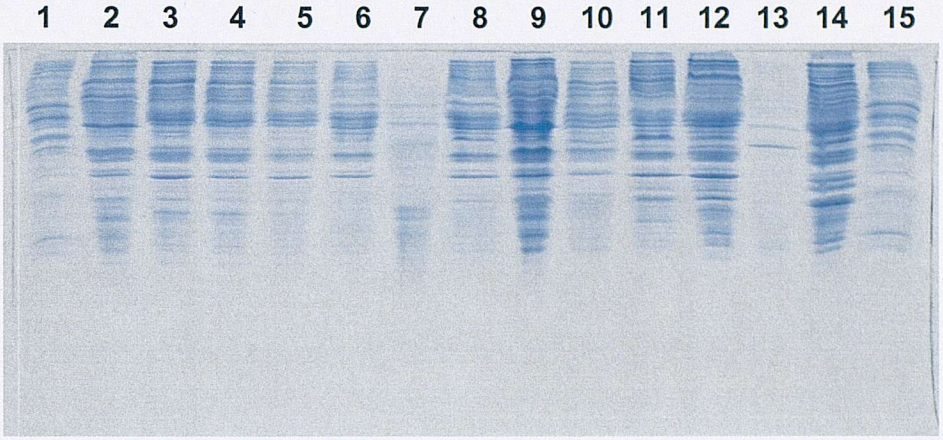
The protein profiles for the novel environmental isolates appeared to be similar except for Lanes 2 (C4DIM), 9 (HWS-01) and 10 (HWS-04). The protein profile for these isolates revealed no similarities in habitats or plant species.



Lane 1 Marker –*Psychrobacter immobilis*, Lane 2 C4DIM- *B. graminis* (*Graminacae* sp.), Lane 3 CWP-01 *Burkholderia* sp. (*Laurus* sp.), Lane 4 CWP-03 *Burkholderia* sp. (*Laurus* sp.), Lane 5 CWS-02 *Burkholderia* sp. (*Quercus* sp.), Lane 6 CWR-04 *Burkholderia* sp. (*Laurus* sp.), Lane 7 YWP-19 *Burkholderia* sp. (*Vaccinium myrtillus*), Lane 8 YWP-21 *Burkholderia* sp.(*Vaccinium myrtillus*), Lane 9 HWS-01 *Burkholderia* sp. (*Salix* sp.), Lane 10 HWS-04 *Burkholderia* sp. (*Fagus* sp.), Lane 11 HWS-21 *Burkholderia* sp.(*Betula* sp.), Lane 12 HWS-29 *Burkholderia* sp. (*Fagus* sp.), Lane 13 HWS-37 *Burkholderia* sp. (*Quercus* sp.), Lane 14 Marker –*P. immobilis*.

Figure 3.6 Protein profiling of *Burkholderia* using SDS PAGE gel electrophoresis: Gel 2 Environmental *Burkholderia* isolates and *B. cepacia* type strain

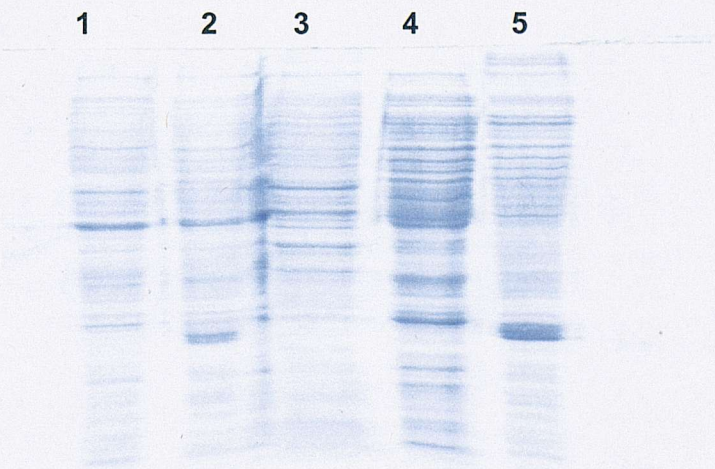
The protein profiles showed no differentiation in habitats or plant species. The protein profile of the onion pathogenic *B. cepacia* type strain 2993 did not reveal a specific band present to differentiate it from the novel isolates.



Lane 1 Marker- *P. immobilis*, Lane 2 HWS-44 *Burkholderia* sp.(*Quercus* sp.), Lane 3 YWS-47 *Burkholderia* sp.(*Betula pendula*), Lane 4 YWS-48 *Burkholderia* sp.(*Betula pendula*), Lane 5 HWS-49 *Burkholderia* sp.(*Betula pendula*), Lane 6 KPS-02 *Burkholderia* sp. (*Quercus* sp.), Lane 7 KPS-05 *Burkholderia* sp. (*Fagus* sp.), Lane 8 OY715 *Burkholderia* sp. (*Allium cepa*), Lane 9 P13F *Burkholderia* sp. (*Quercus* sp.), Lane 10 RW36 *Burkholderia* sp, Lane 11 YWS-01 *Burkholderia* sp. (*Fagus* sp.), Lane 12 YWS-02 *Burkholderia* sp. (*Ulmus* sp.), Lane 13 YWS-03 *Burkholderia* sp. (*Ulmus* sp.), Lane 14 *B. cepacia* type strain 2993 [GIII], Lane 15 Marker –*P. immobilis*.

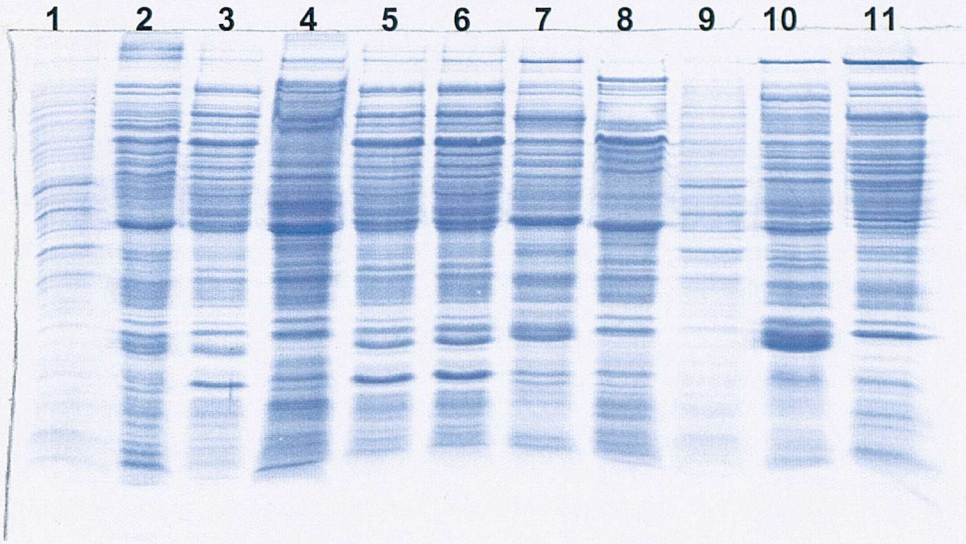
Figure 3.7 Gel 3 Different clinical strains of *B. cepacia*

The profiles for these clinical strains of *Burkholderia* appeared to be very similar.



Lane 1 P3361 *B. cepacia*, Lane 2 P3398 *B. cepacia*, Lane 3 Marker *P. immobilis*, Lane 4 P3399 *B. cepacia*, Lane 5 P3364 *B. cepacia*.

Figure 3.8 Protein profiling of *Burkholderia* using SDS PAGE gel electrophoresis: Gel 4 Different genomovars of *Burkholderia*



Lane 1 *P. immobilis*, Lane 2 P3906 *B. cepacia* [GIII], Lane 3 P3907 *B. stabilis* [GIV], Lane 4 3908 *B. vietnamiensis* [GV], Lane 5 3909 *B. multivorans* [GII], Lane 6 P3910 *B. multivorans* [GII], Lane 7 P3911 *B. cepacia*, Lane 8 P3912 *B. cepacia* [GIII], Lane 9 *P. immobilis*, Lane 10 P3913 *B. vietnamiensis* [GV], Lane 11 *B cepacia* J2315 [GIII].

P- prefix refers to the Protect library, Central Sciences Laboratory, York.

Details in square brackets refers to the genomovar allocation of the isolate.

The various *Burkholderia* species contained within the genomovars could not be differentiated through protein profiling. Lane 2 and 8 were both genomovar III isolates, the protein profiles appear to be different. Lane 5 and 6 contain the protein profiles for two strains *B. multivorans*, the profiles appear to be very similar

3.14 DISCUSSION OF PHENOTYPIC CHARACTERISATION

This section will address the steps taken from the environmental sampling to the production of phenotypic profiles through the use of various classification techniques.

3.14.1 Environmental sampling

The sampling of the novel environmental isolates was carried out over a period of 3 years at several sites within the UK. Environmental factors such as rainfall, light and seasonal conditions were taken into account. It was demonstrated that higher numbers of viable *Burkholderia* isolates were recovered from environmental isolates from the sites which had had rainfall within 24-48h prior to sampling.

From the seasonal aspect the numbers of viable *Burkholderia* were higher during the summer and autumn months when compared to spring. The factors affecting *Burkholderia* recovery may have been due to be moisture levels and availability of nutrients within the soil. During the summer and autumn months there appeared to be a high amount of plant material within the soil from recent seasonal leaf fall. The process of biodegradation from the previous autumn leaf fall, allowed in the nutrients from the plant matter to be released into the soil, thus making them more readily available for bacteria to utilise.

The primary constituent of plant tissue is a complex carbohydrate called cellulose, which is broken down to cellobiose. Other constituents of plant tissue include hemicellulose and pectins that are broken down into sugars such as rhamnose, arabinose and xylose. In previous studies these sugars have been shown to be readily utilised by *Burkholderia* isolates as a carbon source (Gillis *et al.*, 1995; Brett & Waldron, 1996). It is assumed that the *Burkholderia* isolates are able to assimilate the nutrients from the degrading plant matter. It is also assumed that after rainfall the nutrients are made more accessible to the *Burkholderia* isolates within the soil matrix and on the plant rhizospheres. During this study 656 '*Burkholderia*-like' colonies were noted on the initial PCAT isolation plates; a selection were tested, 96 were found to be *Burkholderia* species on the basis of PCR analysis with genus-specific primers. Unfortunately, data on number of colonies from sample plates taken for testing was not recorded at the time of the study. Therefore, it is not possible to examine whether sample type was linked with *Burkholderia* isolation rates, which could have added weight to the habitat or species specificity evidence emerging with the dendrograms.

During the phenotypic characterisation of the novel environmental isolates, the origins of the samples were noted so as to evaluate habitat and plant specificity. Woodland and agricultural areas were sampled throughout this study, numbers of *Burkholderia* appeared to be higher in the woodland areas. The woodland environment provided plenty of plant and tree rhizospheres for competitive colonisation of *Burkholderia* isolates. The rhizosphere provides moisture and nutrients for the growth or maintenance of the residing bacteria (Holmes *et al.*, 1998).

During this study no viable *Burkholderia* isolates were recovered from the samples taken from the agricultural land. This may have been due to the type of soil as the soil sampled was dense and high in clay leaving very little oxygen within the soil matrix. The agricultural soil samples were also taken after the crops were harvested, suggesting that rhizospheric soil was minimal. In addition to this, the soil was taken post-harvest may have had a low nutritional content therefore affecting the bacterial numbers. It has been found that *Burkholderia* can be isolated from the rhizospheres of crop plants such maize (Viallard *et al.*, 1998; Balandreau *et al.*, 2001 and Bevivino *et al.*, 2002) and sugar cane (Brämer *et al.*, 2001). It has also been found that the isolation of *Burkholderia* has been more successful from rhizospheric samples than from bulk soil samples (Hebbar *et al.*, 1992; Di Cello *et al.*, 1997 and Fiore *et al.*, 2001; Richardson *et al.*, 2001).

3.14.2 *Burkholderia* –specific PCR

The use of diagnostic PCR proved to be a useful tool to identify possible *Burkholderia* isolates. The *Burkholderia*-like colonies tested that did not generate a band were assumed to be not from the *Burkholderia* genus. The *Burkholderia*-specific PCR was based on the 16S rDNA gene sequence, which is a highly conserved gene. It was anticipated that the number of false negatives from this screening method would be low due to the highly conserved nature of this gene.

3.14.3 Fatty acid profiling

FAP or fatty acid methyl ester (FAME) analysis has been used to identify bacteria to the genus level, however it has not reliable diagnostically at the species level. It has been previously shown in *Burkholderia* that environmental isolates have different fatty acid profiles to those from clinical samples. In previous work by Jane Richardson, the

presence of the dodecanoic acid (12:0) and tetradecanoic acid (14:0) fatty acid was indicative of woodland *Burkholderia* isolates. In 51 fatty acid profiles of environmental isolates and known *Burkholderia* species, the presence of the fatty acid 12:0 was present in all but 11 woodland isolates.

Some isolates grew poorly on media, which resulted in a large number of plates required for FAP analysis. It appeared that some of the environmental isolates required more than the recommended 24h incubation period to harvest enough cells for this test. An extended incubation period of 48h was used and a comparison on the profiles generated at 24h. During the extended incubation period it was found that the levels of the fatty acid, *cis*-methylene-octadecanoic acid (19:0 cyclo) increased significantly as to affect the eventual identification and clustering of the isolate. Therefore, it was not a viable option to increase the incubation period of the weaker growing isolates to gain more bacterial cells for FAP analysis, as the results could not be compared. Some of the environmental isolates were difficult to maintain on media such as nutrient agar, spontaneous culture death was noted in a number of isolates.

3.14.4 Colonial morphology of novel *Burkholderia* isolates

The colonial morphology of the environmental isolates tended to be very similar to each other, only four isolates were found to produce a yellow pigment on nutrient agar. The novel *Burkholderia* isolates all produced the characteristic 'rotten cabbage' smell generalised for bacteria within this genus. The isolate, CWS-03 produced a yellow pigment similar to the one produced by the onion pathogenic *B. cepacia* type strain 2993. This isolate was also found to induce the symptoms of sour rot in onion tissues. However, the presence of a yellow pigment does not always indicate the onion pathogenicity of the isolate.

Growth at 37°C was previously used as an indicator of possible human pathogenicity of *Burkholderia* isolates. A small selection of novel *Burkholderia* isolates was selected and out of 16 isolates 6 showed good signs of growth at 37°C whilst 2 showed weak signs of growth. All of the novel isolates grew well at 28°C with 8 failing to grow at 37°C. The ability to grow at 37°C could only be used to indicate the potential for human colonisation by the novel isolate. Growth at 37°C was not an absolute indicator for causing morbidity or mortality in human hosts, other characteristics such as virulence genes need to be considered. The clinical epidemic strain *B. cepacia* J2315

also demonstrated an ability to grow at 28°C thus demonstrating that it may have the ability to survive outside a human host.

It is stated in a study by Yohalem & Lorbeer (1997) that the maceration of onion tissue was indicative to the plant pathogenicity of the *Burkholderia* isolate. At present, clinical strains have not shown a cross pathogenicity between the human and plant hosts. However, concerns have arisen in regard to clinical isolates possessing some similar 'physical' characteristics to environmental strains. For example, the epidemic strain *B. cepacia* J2315 has been found to be capable of macerating onion tissue so as to cause the symptoms of sour rot (Butler *et al.*, 1995). Fortunately, this strain of *B. cepacia* can not be readily found within the environment where it would be a problem in relation to agriculture and human health. Converse to this finding is that some environmental strains of *B. cepacia* have been found to cause human infection. This has been illustrated with the incidence of 'foot rot' in army troops training in swamps in Florida, USA (Taplin *et al.*, 1971). Hence, there are grave concerns that phenotypic characteristics may not be enough to gauge the potential for plant or human pathogenicity. Further study will be required to devise more robust and reproducible methods to investigate the possible transmission of genes between pathogenic and non-pathogenic strains of *Burkholderia*.

3.14.5 Carbohydrate metabolism of novel *Burkholderia* species

From previous studies it has been suggested that *Burkholderia* isolates are nutritionally diverse. The use of commercial systems such as Phenotypic Microarrays™ (Biolog) or API strips (BioMérieux) for the characterisation of *Burkholderia* isolates *via* 'metabolic fingerprints' has proved to be reliable to the genus level but unreliable in species identification. However, it gives a useful insight to how different environmental habitats may affect carbohydrate profiles of novel *Burkholderia* isolates.

The outcome from the API 20NE carbohydrate utilisation test confirmed that there was no common carbohydrate profile for woodland isolates. However, there was a common inability to produce a positive result / breakdown / utilisation for the following agents reduction of nitrates to nitrogen (N_2), tryptophane (TRP), glucose (GLU) acidification and arginine (ADH). The novel isolates also were shown to have variable oxidase activity. The API 50CH test did not generate a common carbohydrate profile

for the novel *Burkholderia* isolates. However, the inability to utilise α-methyl-D-mannoside (α-MDM), α-methyl-D-glucosamine (α-MDG), N-acetyl-glucosamine (NAG), amygdalin (AMY), starch (STA), glycogen (GLYG), xylitol (XLT), gentiobiose (GEN), gluconate (GNT), 2-keto-gluconate (2KG) and 5-keto-gluconate (5KG) was found in all of the isolates tested.

The nutritional diversity of *Burkholderia* may also gauge how useful the isolate is for beneficial practices such as bioremediation. In several studies it has been shown that some *Burkholderia* isolates have the ability to break down and utilise complex chemical compounds (McGowan *et al.*, 1998; Masters & Mohn, 2001; Nogales *et al.*, 2001; Rapp, 2001 and Seah *et al.*, 2001). Should *Burkholderia* isolates utilised as bioremedial agents either as a single culture or forming part of a consortium of bacteria; it may be able to utilise a range of metabolites produced from the remediation of the complex chemical compounds.

3.14.6 Protein profiles of *Burkholderia* isolates

From the visual analysis of the protein profiles no clusters containing woodland isolates could be identified. The onion pathogenic strains, OY715 and *B. cepacia* type strain 2993 had very different protein profiles to each other. There were no common bands present within these two profiles that would distinguish them from the non-plant pathogenic strains. Therefore, suggesting that there may not be a common protein profile for plant pathogenicity.

The protein profiles also failed to categorise the different genomovars into distinct groups (dendrogram not provided). The profiles produced by the two *B. cepacia* genomovar III isolates (P3906 and P3912) appeared to be different. However, the two *B. multivorans* strains appeared to be very similar to each other. The two *B. vietnamiensis* strains (P3908 and P3913) profiles differed; it was expected that these two strains should have clustered closely. This showed that protein profiling when used as a taxonomic tool could not successfully group *Burkholderia* according to species.

There appeared to be a strong correlation of the protein bands from the isolates from specific habitats. However, the differences in band generation found between the various habitats were not great enough to form distinct clusters. There also seemed to

be a lack of reproducibility using protein profiles as the marker strains to align the protein gel strips as they appeared to differ from each other. This event suggested that replication of protein profiles from the same isolate could lead to a varying patterns being generated. This finding was confirmed in 1998 by work carried out in by Stead *et al.*, investigating methods for identifying bacteria from plant samples (Stead *et al.*, 1998).

Protein profiling and FAP profiling could be useful techniques in the initial genus characterisation of bacteria. However, another method or a combination of methods will be required for the accurate taxonomy of novel *Burkholderia* environmental isolates. Only through the combination of methods can a higher level of discrimination be achieved with this genus of bacteria.

3.14.7 Speciation of novel *Burkholderia* isolates

None of the phenotypic classification methods used during this chapter were effective in the speciation of the novel isolates. The phenotypic profiles were so diverse that plant species specific clusters could not be formed. However, if this work was carried out using a panel of known *Burkholderia* species it may be possible to identify common profiles between the novel isolates. Due to time and financial constraints, this work was not carried out as a part of this study.

CHAPTER 4

GENOTYPIC CHARACTERISATION OF NOVEL *BURKHOLDERIA* ISOLATES

4.0 Background

This chapter will use a number of molecular methods to classify the novel and fully characterised *Burkholderia* into meaningful groups. The phenotypic profiles generated from chapter 3 will also be used in combination with the genotypic profiles generated to create a polyphasic taxonomic grouping of the novel *Burkholderia* isolates.

4.1 BOX PCR analysis of novel environmental isolates of *Burkholderia*

The BOX element PCR is a method of generating whole genome fingerprints of micro-organisms through targeting repetitive sequences found throughout the genome. This method was used in previous work carried out by Jane Richardson as part of her PhD studies and it has grouped some members of the *Burkholderia* genus. BOX element PCR was chosen to gather further genetic information to be combined with the work already carried out. Further to this, the BOX element PCR had to be tested for its robustness between operators and at different time points.

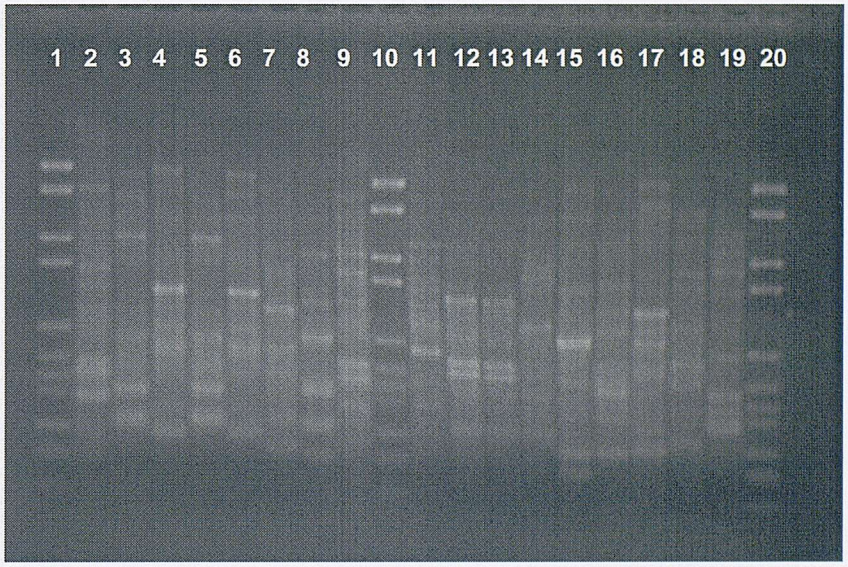
Novel *Burkholderia* isolates from the York and Hampstead Heath woodlands, type strains, other *Burkholderia* species and clinical isolates were included in this analysis. The various characteristics such as plant pathogenicity, human pathogenicity, environmental and rhizospheric strains were represented as a part of this analysis.

The *Burkholderia graminis* strains (C3AIM, C4DIM and C5AIM) were obtained from *Graminacae* sp. (maize) rhizospheres and provided by Dr Jacques Balandreau, France.

Figure 4.1 BOX PCR of novel environmental isolates of *Burkholderia*

This gel shows the BOX DNA fingerprints generated from 17 novel *Burkholderia* isolates. A total of 41 novel *Burkholderia* isolates were eventually genotypically profiled using this method (these profiles can be found in figure 4.5). This number of novel isolates differed from the original 96 confirmed *Burkholderia* isolates mentioned in Chapter 3 due to the cultures not being able to survive on artificial media or when stored at -80°C in glycerol.

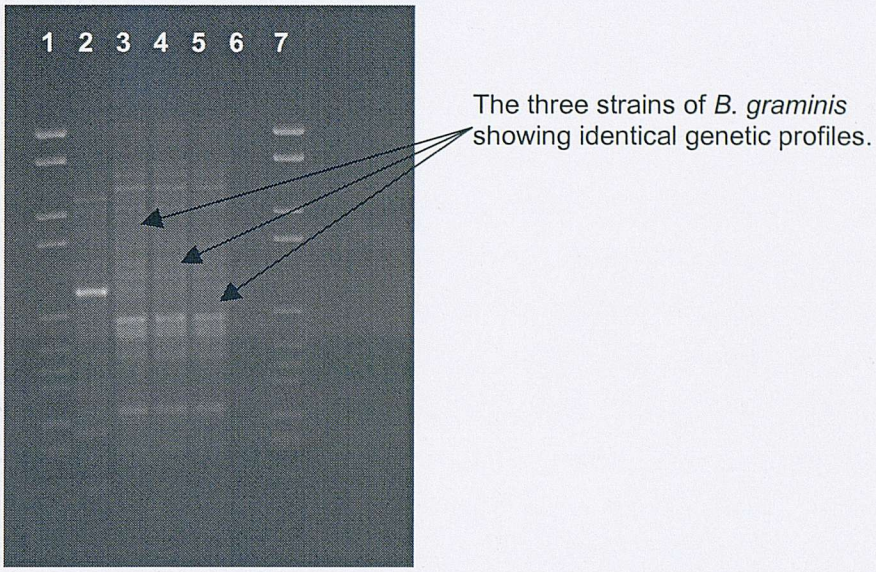
This gel represents some of the BOX fingerprints used to create a dendrogram. The dendrogram illustrates the relatedness of each of the novel isolates to fully characterised *Burkholderia* species (see figure 4.5 for the BOX dendrogram).



Lane 1 Marker VI (Boeringer-Mannheim) Lane 2 HWP-12 *Burkholderia* sp. (*Fagus* sp.), Lane 3 YWP-18 *Burkholderia* sp. (*Vaccinium myrtillus L.*), Lane 4 YWP-19 *Burkholderia* sp. (*Vaccinium myrtillus L.*), Lane 5 YWP-20 *Burkholderia* sp. (*Vaccinium myrtillus L.*), Lane 6 YWP-21 *Burkholderia* sp. (*Vaccinium myrtillus L.*), Lane 7 YWP-22 *Burkholderia* sp(*Vaccinium myrtillus L.*), Lane 8 HWR-10 *Burkholderia* sp. (Unknown plant root), Lane 9 HWP-15 *Burkholderia* sp. (*Quercus* sp.), Lane 10 Marker VI, Lane 11 HWP-20 *Burkholderia* sp. (*Fagus* sp.), Lane 12 HWS-01 *Burkholderia* sp. (*Betula pendula*), Lane 13 HWS-04 *Burkholderia* sp. (*Fagus* sp.), Lane 14 HWP-12 *Burkholderia* sp. (*Fagus* sp.), Lane 15 HWS-21 *Burkholderia* sp. (*Betula* sp.), Lane 16 HWS-25 *Burkholderia* sp. (*Betula* sp.), Lane 17 HWS-27 *Burkholderia* sp. (*Fagus* sp.), Lane 18 HWS-29 *Burkholderia* sp. (*Fagus* sp.), Lane 19 HWS-32 *Burkholderia* sp. (*Quercus* sp.), Lane 20 Marker VI.

Figure 4.2 BOX PCR fingerprints of *Burkholderia* species

This gel image shows the BOX DNA fingerprints from a strain of *B. gladioli* and 3 strains of *B. graminis.* The three *B. graminis* strains display very similar DNA fingerprint profiles.



Lane 1 and 7 Marker VI (Boeringer-Mannheim), Lane 2 P1970 (*B. gladioli* pv. *allicola*), Lane 3 C3AIM (*B. graminis*), Lane 4 C4DIM (*B. graminis*), Lane 5 C5AIM (*B. graminis*), Lane 6 Negative control.

The gel images were transferred onto an analytical computer program (GelCompar version 4.0) to calculate the similarities between each set of fingerprints generated from the BOX PCR. The fingerprint data from the gels were then combined to create a dendrogram (see figure 4.5). The formation of the cophenetic value indicated the stability of the dendrogram. It suggests the probability of the individual fingerprints clustering together, the higher the value the more stable the dendrogram is.

Previous BOX PCR data were also included in the dendrogram. The additional data were generated from Jane Richardson as a part of her PhD studies. Inclusion of these data would address the reproducibility of the of BOX PCR fingerprints through different operators. The fingerprint profiles been able to be aligned closely to each other would indicate that BOX PCR is a robust method for taxonomic studies.

4.2 Analysis of BOX PCR dendrogram of all *Burkholderia* isolates- Time and Operator differences

Internal '*Burkholderia* strain controls' were included to monitor the compatibility of the two sets of BOX fingerprint data. The '*Burkholderia* strain controls' consisted of identical strains which were tested and analysed by two separate operators. Through the inclusion of the controls, it became evident that the two sets of data were significantly different from each other. Therefore, the novel isolates recovered from this study could not be compared directly with the work carried out by Jane Richardson (figure 4.3). This finding suggested that the BOX PCR method introduced another variable when operated by two individuals at different occasions, thus making the data difficult to compare. When BOX PCR analysis was carried out by a single operator, there appeared to be some variation in the fingerprint profiles generated (Figure 4.4). It was therefore important to carry out all of the fingerprints at the same time in order to reduce as much variation as possible within the fingerprints generated. The DNA extracts once prepared, had to be used for the BOX and ERIC PCR amplification analyses within 48 h for optimal results.

Figure 4.3 Dendrogram of BOX DNA fingerprints from *Burkholderia* species – Operator differences

Cophenetic correlation of 86.6%

(The asterisks * were used to distinguish the BOX fingerprints generated during the course of this study from those previously produced by Jane Richardson to demonstrate the introduction of changes of DNA profile due to different operators.)

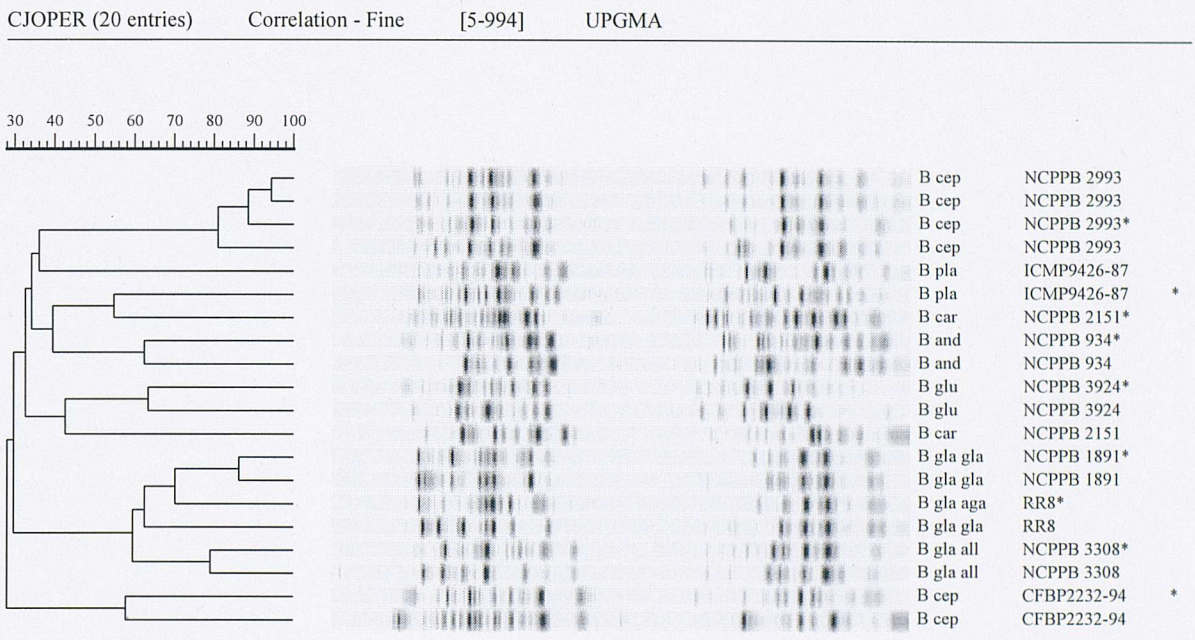


Figure 4.4 Dendrogram of BOX DNA fingerprints from novel woodland isolates and *Burkholderia* species – Time differences

Cophenetic correlation of 88.0%

This dendrogram shows that the fingerprints differed slightly over time with the *B. cepacia* type strain 2993. Some of the novel woodland isolates displayed a large change in fingerprint profiling over time. This can be seen in CWP-01 and CWS-02 for example.

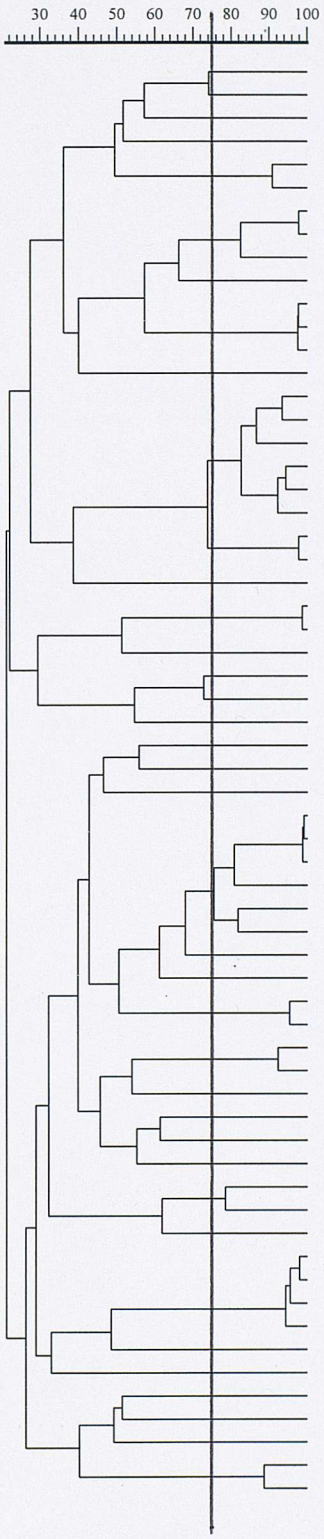
(the asterisks * were used to distinguish the novel woodland isolates tested for the final BOX fingerprint dendrogram from those tested at earlier dates)

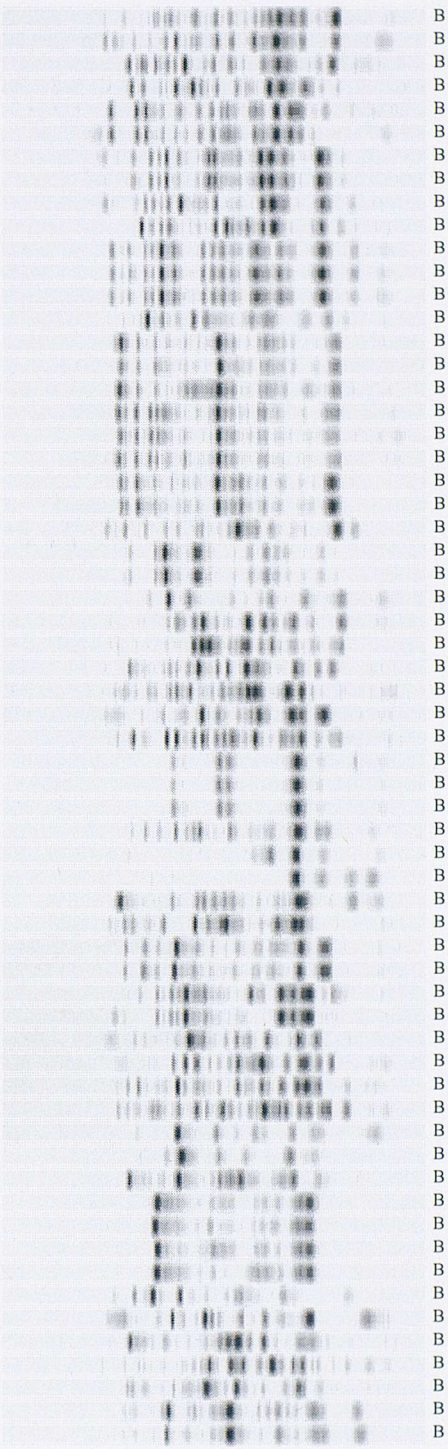


Figure 4.5 Dendrogram of BOX DNA fingerprints from novel woodland isolates and *Burkholderia* species

Cophenetic correlation of 75.3%

(The asterisks * were used to distinguish the novel woodland isolates and various *Burkholderia* species used during this study from those collected previously by Jane Richardson. See Appendix A12 for combined dendrogram containing novel isolates and Jane Richardson's collection).





B sp	HWS-25*	Birch	
B sp	CWS-02*	Oak	
B amb	LMG 19182*	Rhizosphere	
B sp	HWR-15*	Plant root	
B sp	HWR-10*	Plant root	
B sp	HWL-10*	Plant	
B sp	HWS-01*	Willow	1
B sp	HWS-04*	Beech	
B sp	CWR-01*	Oak	
B sp	CWR-03*	Sycamore	
B gra	C4DIM*	Maize	2
B gra	C5AIM*	Maize	
B gra	C3AIM*	Maize	
B sp	CUP*	Plant	
B sp	YWP-21*	Bilberry	3
B sp	YWP-19*	Bilberry	
B sp	YWS-05*	Ash	
B sp	YWS-48*	Silverbirch	
B sp	YWS-49*	Silverbirch	
B sp	YWS-47*	Silverbirch	
B sp	HWS-27*	Woodland	
B sp	HWS-29*	Beech	
B sp	HWS-21*	Birch	
B cal	LMG 19076*	Rhizosphere	
B fun	LMG 16225*	Fungus	
B cep	LMG 18863*	CF	
B sp	CWR-02*	Oak	
B sp	CWS-03*	Sycamore	
B pla	ICMP 9426-87	*	
B car	NCPPB 2151*		
B and	NCPPB 934*		
B sp	HWR-20*	Plant root	
B mul	LMG 16660*	CF	4
B mul	LMG 13010*	CF	
B sta	LMG 18888*	Human Blood	
B sp	CWP-03*	Laural	
B cep	LMG 16654*	CF	
B cep	LMG 16659*	CF	
B sp	CWR-04*	Laural	
B sp	YWS-46*	Silverbirch	
B sp	YWP-18*	Bilberry	
B sp	YWP-20*	Bilberry	
B sp	HWS-32*	Oak	
B sp	HWS-34*	Sycamore	
B glu	NCPPB 3924*		
B sp	CWP-01*	Laural	
B sp	CWP-02*	Plant	
B sp	YWP-22*	Bilberry	
B vie	LMG 10929*	Rhizosphere	
B vie	LMG 18836*	CGD patient	
B gla gla	NCPPB 1891*		
B sp	HWS-37*	Oak	5
B sp	CWS-01*	Oak	
B sp	HWS-44*	Oak	
B sp	P13F*	Oak	
B sac	LMG 19450*	Sugar cane	
B cep	CFBP 2232-94	* Clinical	
B gla aga	RR8*	Mushroom	
B sp	CWR-06*	Ash	
B cep	NCPPB 2993*	Onion	
B gla all	NCPPB 3308*		
B sp	CWL-01*	Leaf	

4.3 Analysis of BOX PCR dendrogram of novel *Burkholderia* isolates 1999-2001

Within this dendrogram (figure 4.5) 62 *Burkholderia* species and novel isolates were tested to investigate the presence of plant specificity, habitat specificity, plant pathogenicity and human pathogenicity. Not all of the novel *Burkholderia* isolates found in the previous chapter were tested using this method. This was due to some isolates being unable to survive in the 'artificial environment' provided by the growth media.

As an indication of homology and relatedness, 75% correlation of fingerprint profiles was used to identify the clusters. This helped to define closely related clusters and to highlight any groups of interest. The formation of the cophenetic value indicated the stability of the dendrogram. This value indicates the probability of individual fingerprints clustering together, the higher the value the more stable the dendrogram is.

As seen from the dendrogram (figure 4.5) there are 5 distinct clusters (one of which contained only clinical isolates) formed from this analysis. Cluster 1 contained a variety of isolates from different tree species, this cluster did not exhibit any signs of plant specificity within it. Cluster 2 contained only the *B. graminis* strains (C3AIM, C4DIM and C5AIM); these strains displayed 97.5% homology to each other, some plant specificity can be seen from this cluster.

Cluster 3 contained isolates from rhizospheric and rhizospheric soil samples of trees and plants. This particular cluster did not show plant species or habitat specificity, as the isolates originated from Sand Hutton and Hampstead Heath sites. However, it should be noted that all of the *Betula pendula* (silver birch) isolates were contained within this cluster. This may have indicated a degree of either tree species specificity or habitat specificity as all of these isolates were also from the same woodland site.

Cluster 4 contained only clinical or human isolates of *B. multivorans* or *B. stabilis*. These two species have been described to be very similar to each other in previous work carried out by Jane Richardson using this method (see appendix A12). Therefore, it was not unusual to find these two species clustered closely with each other within this dendrogram. Cluster 5 contained only *Quercus* sp. (oak) isolates which also suggested a plant species specificity developing. However, four other

Quercus sp. (oak) isolates could also be found elsewhere within the dendrogram, one of these isolates was found to be grouped with beech and willow isolates in Cluster 1.

The human isolates failed to form any kind of cluster and all were found scattered throughout the dendrogram. Despite the failure of the human isolates grouping together, the two *B. multivorans* isolates were found within the same cluster (Cluster 4). This suggested a degree of species specificity of this method for the grouping of the *B. multivorans* isolates.

4.4 ERIC PCR analysis of novel environmental isolates of *Burkholderia*

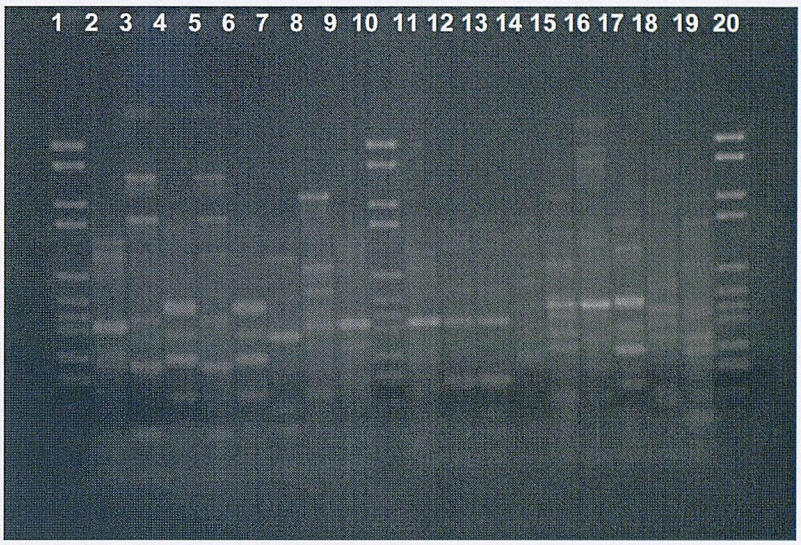
ERIC PCR was another method of generating whole genome DNA profiles. This taxonomic method uses a repetitive region called ERIC which can be found throughout the genome. This method was also used by Jane Richardson in part of her PhD studies attempt to group the members of the genus *Burkholderia* and her novel isolates. Through combining the pre-existing ERIC fingerprint data with the novel isolates acquired during the course of this study, it was hope that the robustness of this method could be investigated.

Sixty two novel environmental isolates from the woodland sites, type strains, other *Burkholderia* and three isolates of *Burkholderia graminis* were included for fingerprinting by ERIC PCR (figures 4.6 and 4.7 show examples of ERIC fingerprints generated by novel woodland isolates). Only 42 novel woodland isolates were tested as a large number of novel *Burkholderia* isolates could not survive on artificial media or storage at -80°C, therefore they were subsequently not available for this analysis.

As with the BOX PCR analysis, the characteristics of plant pathogenicity, human pathogenicity, environmental and rhizospheric strains were investigated. The fingerprints generated from this and other batches of ERIC PCR amplifications were then entered into a database and compared with each other through the use of GelCompar version 4.0 software (Figure 4.8). As previously mentioned, all of the ERIC PCR fingerprints were generated on the same day in order to remove as much time variability and increase comparability.

Figure 4.6 ERIC DNA fingerprints of novel woodland *Burkholderia* isolates

This gel shows an example of the ERIC fingerprints generated from some of the novel woodland *Burkholderia* isolates. The ERIC fingerprints were then subsequently compared using the GelCompar software to observe any cluster formations amongst the novel woodland isolates.



Lane 1 Marker VI (Boeringer-Mannheim) Lane 2 HWP-12 *Burkholderia* sp. (*Fagus* sp.), Lane 3 YWP-18 *Burkholderia* sp. (*Vaccinium myrtillus L.*), Lane 4 YWP-19 *Burkholderia* sp. (*Vaccinium myrtillus L)*, Lane 5 YWP-20 *Burkholderia* sp. (*Vaccinium myrtillus L.*), Lane 6 YWP-21 *Burkholderia* sp. (*Vaccinium myrtillus L.*), Lane 7 YWP-22 *Burkholderia* sp. (*Vaccinium myrtillus L.*), Lane 8 HWR-10 *Burkholderia* sp. (Unknown plant), Lane 9 HWR-15 *Burkholderia* sp. (Unknown plant root), Lane 10 Marker VI, Lane 11 HWS-02 *Burkholderia* sp. (*Betula pendula*), Lane 12 HWS-01 *Burkholderia* sp. (*Betula pendula*), Lane 13 HWS-04 *Burkholderia* sp. (*Fagus* sp.), Lane 14 HWS-20 *Burkholderia* sp. (*Betula* sp.), Lane 15 HWS-21 *Burkholderia* sp. (*Betula* sp.), Lane 16 HWS-25 *Burkholderia* sp. (*Betula* sp.), Lane 17 HWS-27 *Burkholderia* sp. (*Fagus* sp.), Lane 18 HWS-29 *Burkholderia* sp. (*Fagus* sp.), Lane 19 HWS-32 *Burkholderia* sp. (*Quercus* sp.), Lane 20 Marker VI.

Figure 4.7 ERIC DNA fingerprints of *Burkholderia* species

The gel shows the example of ERIC fingerprints generated from various species of *Burkholderia,* including three strains of *B. graminis*. The 3 strains of *B. graminis* show very similar ERIC fingerprint profiles (see lanes 7, 9 and 10). Again, the 3 *B. graminis* strains (C3AIM, C4DIM and C5AIM) have shown to possess similar DNA fingerprint profiles. The *B. gladioli* strains (lanes 2, 3, 4 and 6) have also show a similarity in fingerprint profiles; to show a degree of species grouping through use of this method.



Lane 1 DNA Marker VI (Boeringer-Mannheim), Lane 2 165 (*B. gladioli* pv *allicola*), Lane 3 410 / RR8 (*B. gladioli* pv *agaricola*), Lane 4 P1970 (*B. gladioli* pv *allicola*), Lane 5 2993 (*B. cepacia* type strain), Lane 6 3207a (*B. gladioli*), Lane 7 C3AIM (*B. graminis*), Lane 8 Marker VI, Lane 9 C4DIM (*B. graminis*), Lane 10 C5AIM (*B. graminis*).

4.5 ERIC analysis of novel isolates of *Burkholderia*

As a part of this study it was intended to seek specificity using this particular method the results of which can be seen in Figure 4.8. It was hoped that host or habitat specificity, type of pathogenicity, bioremedial potential would be revealed through the clustering of isolates. Alongside the novel isolates, type strains of *Burkholderia* species were used to enable a comparison of the novel isolates to pre-characterised species. It was hoped that phenotypic characteristics of the genotypic groupings can be shown through the use of this method.

Using 75% as the level of homology, 5 distinct clusters within this dendrogram were identified. None of the 5 clusters formed displayed exclusive specificity for clinical isolates within the genus *Burkholderia*. However, there appeared to be a formation of a cluster containing *Quercus* sp. (oak) rhizospheric samples (cluster 2). These *Quercus* sp. isolates were obtained from separate trees from geographically distinct sites, which may show species specificity rather than geographical / habitat specificity.

The clinical isolates *B. multivorans* LMG 16660, LMG 13010 and *B. stabilis* LMG 18888 formed a small cluster (cluster 3) with homology reaching as high as 97%. Cluster 5 contained rhizospheric isolates from various trees and plants. However, the same type of isolates could be found elsewhere within the dendrogram. Again, the specificity towards habitat or plant could not be found as other isolates from the same sample types were scattered throughout the dendrogram. Through combining all of the fingerprints into a single dendrogram, one of the *B. graminis* strains (C5AIM) appeared to not be clustered as closely to the other two strains.

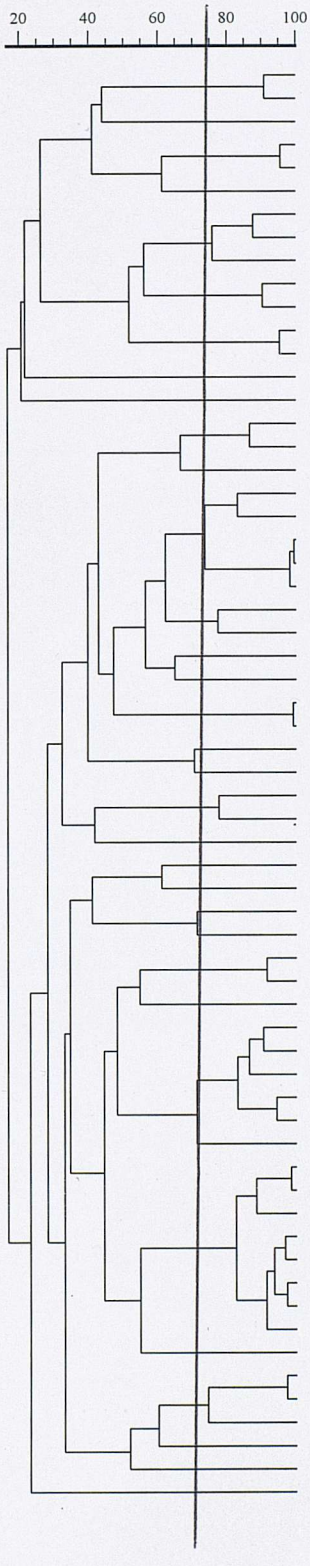
4.6 Analysis of ERIC and BOX DNA fingerprints of *Burkholderia* species and novel isolates – Operator differences

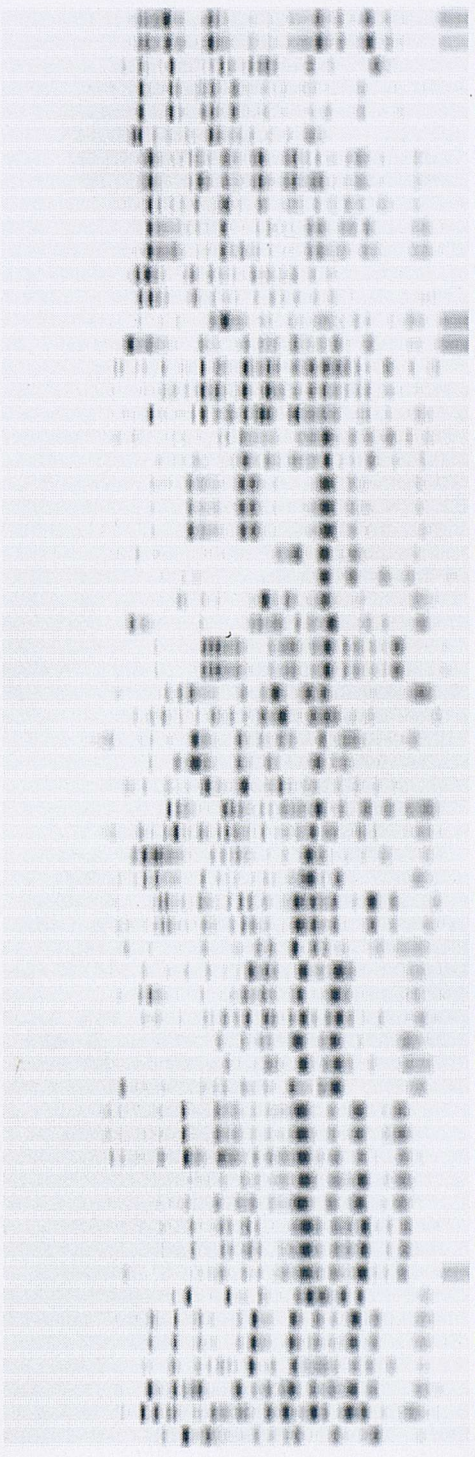
The BOX and ERIC data generated by Jane Richardson as part of her PhD studies were combined with the novel fingerprints from the woodland isolates were included with the novel woodland isolates (see appendix A12 for combined ERIC fingerprint dendrogram). The results demonstrated that there were differences between the fingerprints generated and several bands were either missing or present as a result from the techniques adopted by the two operators. The data from the two sets of BOX and ERIC PCR amplifications could not be compared on an equal basis to one another in an effort to achieve a good phylogenetic comparison of the novel isolates with pre-existing data. However, an assumption on habitat specificity could be made from the combined data. This assumption can be shown in the loose grouping of the woodland isolates of Jane Richardson to the novel woodland isolates obtained during this study. However, the level of relatedness of Jane Richardson's isolates and the novel woodland isolates was at 62%. Taking the effects of operator differences the clustering of the woodland isolates could be at a higher (or lower) level of relatedness if the ERIC fingerprints were generated at the same time.

Figure 4.8 ERIC DNA fingerprint dendrogram of *Burkholderia* species and novel isolates

Cophenetic correlation of 77.6%

(The asterisks * were used to distinguish the novel woodland isolates and various *Burkholderia* species used during this study from those collected previously by Jane Richardson. This marking system can be found in use within the combined operator ERIC dendrograms, see appendix A12)





B sp	YWP-18*	Bilberry	] 1
B sp	YWP-20*	Bilberry	
B sac	LMG 19450*	Sugar cane	
B cal	LMG 19076*	Rhizosphere	
B fun	LMG 16225*	Fungus	
B amb	LMG 19182*	Rhizosphere	
B sp	HWS-44*	Oak	] 2
B sp	P13F*	Oak	
B sp	HWS-37*	Oak	
B sp	HWS-32*	Oak	
B sp	HWS-34*	Sycamore	
B sp	CWP-01*	Laural	
B sp	CWR-02*	Oak	
B sp	CWP-03*	Laural	
B sp	CWP-02*	Plant	
B sp	CWR-06*	Ash	
B sp	CWS-03*	Sycamore	
B sp	CWS-01*	Oak	
B sp	HWR-15*	Root	
B sp	HWR-20*	Root	
B mul	LMG 16660*	CF	] 3
B mul	LMG 13010*	CF	
B sta	LMG 18888*	Human blood	
B cep	LMG 16654*	CF	
B cep	LMG 16659*	CF	
B cep	NCPPB 2993*	Onion	
B sp	CWR-04*	Laural	
B sp	HWS-01*	Willow	
B sp	HWS-04*	Beech	
B sp	YWS-46*	Silverbirch	
B sp	CWR-01*	Oak	
B vie	LMG 10929*	Rhizosphere	
B vie	LMG 18836*	Rhizosphere	
B glu	NCPPB 3924*		
B and	NCPPB 934*		
B sp	CUP*	Plant	
B sp	HWS-25*	Birch	
B sp	CWS-02*	Oak	
B sp	HWR-10*	Root	
B sp	HWL-10*	Leaf	
B cep	CFBP 2232-94	* Clinical	
B gla all	NCPPB 3308*		] 4
B sp	CWL-01*	Leaf	
B sp	CWR-03*	Sycamore	
B gla gla	NCPPB 1891*		
B gla aga	RR8*	Mushroom	
B sp	YWP-22*	Bilberry	] 5
B sp	YWS-47*	Silverbirch	
B sp	YWS-48*	Silverbirch	
B sp	YWS-49*	Silverbirch	
B sp	YWP-21*	Bilberry	
B sp	YWS-05*	Ash	
B sp	HWS-27*	Beech	
B sp	HWS-29*	Beech	
B sp	YWP-19*	Bilberry	
B sp	HWS-21*	Woodland	
B gra	C3AIM*	Maize	
B gra	C4DIM*	Maize	
B pla	ICMP 9429-87	*	
B gra	C5AIM*	Maize	
B car	NCPPB 2151*		
B cep	LMG 18863*	CF	

4.7 ERIC DNA fingerprint dendrogram of *Burkholderia* species and novel isolates

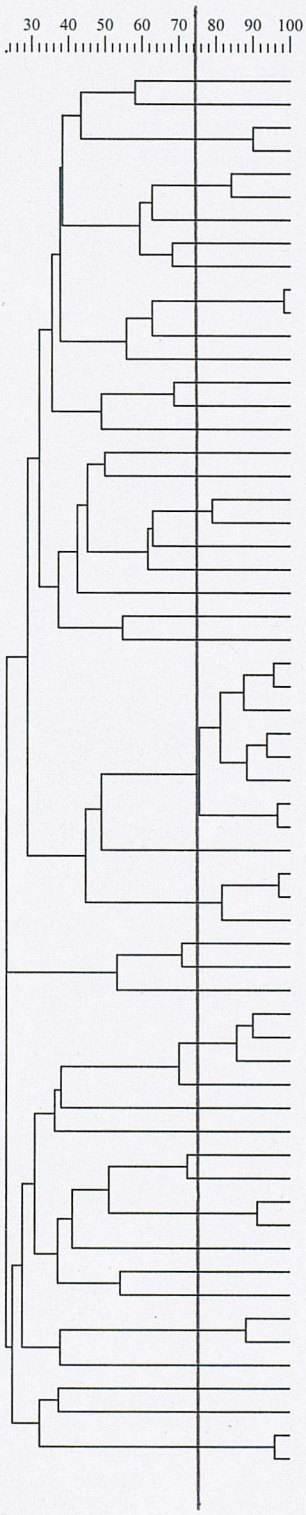
The ERIC DNA fingerprint dendrogram revealed a limited clustering according to plant species, woodland habitat or type of pathogenicity (human or plant). (see figure 4.8). The woodland isolates failed to cluster together and could be found dispersed throughout the dendrogram. The ERIC analysis failed to generate any form of species identification for the novel *Burkholderia* isolates. As previously stated by Jane Richardson the only species which clustered together in this analysis were the *B. gladioli* and *B. andropogonis* species. The addition of more type strains and novel isolates for analysis has resulted in the appearance of some novel clusters which are habitat related. For instance a cluster of *B. multivorans* (clinical isolates), has been identified but since only 2 strains were used in the analysis, the assumption that cluster 1 is specific for this species is premature, especially as a *B. stabilis* isolate can also be found within this cluster.

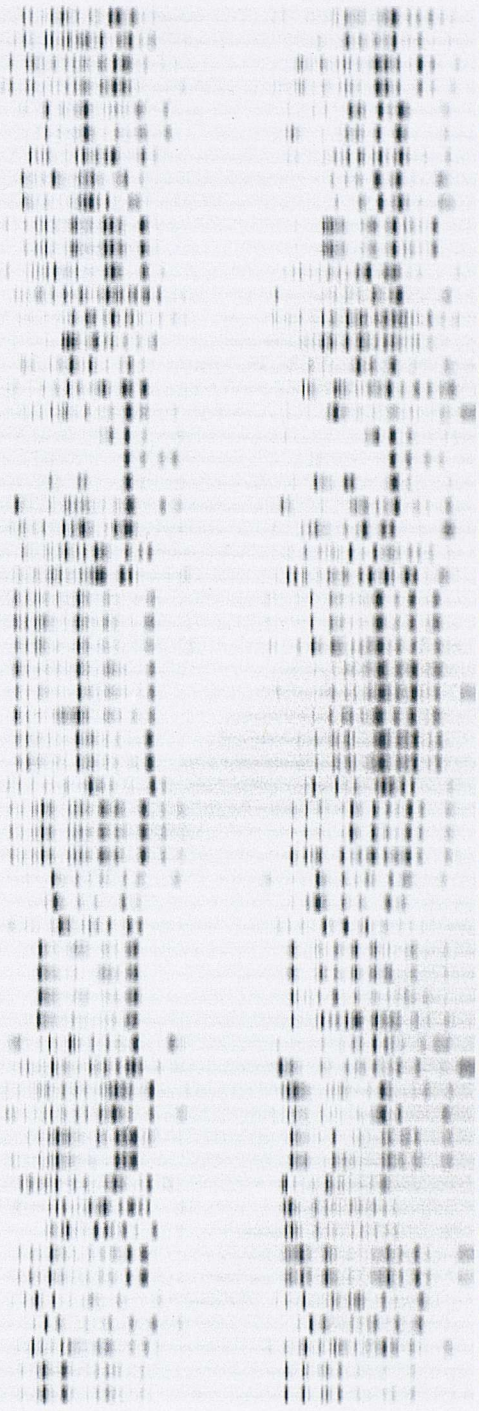
Figure 4.9 BOX-ERIC combined dendrogram data from novel woodland isolates and *Burkholderia* species

Cophenetic correlation of 75.0%

(The asterisks * were used to distinguish between the novel woodland isolates collected during this study from those collected previously by Jane Richardson.)

(See Appendix A12 for combined BOX-ERIC data containing Jane Richardson's data)





B sp	HWR-15*	Plant root	
B sp	HWR-20*	Plant root	
B sp	HWR-10*	Plant root	
B sp	HWL-10*	Plant	
B gla all	NCPPB 3308*		
B sp	CWL-01*	Leaf	
B sp	CWR-03*	Sycamore	
B gla gla	NCPPB 1891*		
B gla aga	RR8*	Mushroom	
B sp	HWS-01*	Woodland	
B sp	HWS-04*	Beech	
B sp	CWR-01*	Oak	
B sp	YWP-22*	Bilberry	
B sp	CWR-06*	Ash	
B sp	CWS-03*	Sycamore	
B cep	NCPPB 2993*	Onion	
B and	NCPPB 934*		
B sp	CWP-03*	Laural	
B cep	LMG 16654*	CF	
B cep	LMG 16659*	CF	
B sta	LMG 18888*	Human blood	
B sp	CWR-04*	Laural	
B sp	YWS-46*	Silverbirch	
B pla	ICMP9426-87	*	
B car	NCPPB 2151*		
B sp	YWS-47*	Silverbirch	1
B sp	YWS-48*	Silverbirch	
B sp	YWS-49*	Silverbirch	
B sp	YWP-21*	Bilberry	
B sp	YWP-19*	Bilberry	
B sp	YWS-05*	Ash	
B sp	HWS-27*	Woodland	
B sp	HWS-29*	Beech	
B sp	HWS-21*	Birch	
B gra	C3AIM*	Maize	2
B gra	C4DIM*	Maize	
B gra	C5AIM*	Maize	
B vie	LMG 10929*	Rhizosphere	
B vie	LMG 18836*	CGD patient	
B glu	NCPPB 3924*		
B sp	HWS-44*	Oak	3
B sp	P13F*	Oak	
B sp	HWS-37*	Oak	
B sp	CWS-01*	Oak	
B cep	CFBP2232-94	* Clinical	
B sp	CWP-02*	Plant	
B sp	HWS-25*	Birch	
B sp	CWS-02*	Oak	
B sp	HWS-32*	Oak	
B sp	HWS-34*	Sycamore	
B amb	LMG 19182*	Rhizosphere	
B sp	CWP-01*	Laural	
B sp	CWR-02*	Oak	
B sp	YWP-18*	Bilberry	
B sp	YWP-20*	Bilberry	
B sac	LMG 19450*	Sugar cane	
B cep	LMG 18863*	CF	
B sp	CUP*	Plant	
B cal	LMG 19076*	Rhizosphere	
B fun	LMG 16225*	Fungus	

4.8 BOX-ERIC combined dendrogram data- Operator differences

The robustness of the BOX-ERIC dendrogram was investigated through combining two data sets from separate operators. The BOX-ERIC dendrogram contained within appendix A12 highlighted the differences the *B. cepacia* type strain 2993 fingerprints varied from 5-20% between the operators. On further analyses, other isolates showed differences between each other to an extent that they could potentially be defined as different species. For instance, the variations in the fingerprint profiles for different isolates of *B. plantarii, B. andropogonis, B. glumae* and *B. gladioli* were only at a level of 30% homology. The reasons for these differences are multifarious but culture age and operator variations are clearly involved. For example, the generation or removal of just one band from the fingerprint containing 20 bands was sufficient to separate it from the other isolates contained within the dendrogram by 5%.

4.9 Combined BOX-ERIC data dendrogram of novel *Burkholderia* isolates

Through the combination of these two PCR methods, an increased level of discrimination can be used to differentiate between the *Burkholderia* species and novel isolates contained within it (see figure 4.9).

The combined BOX-ERIC dendrogram failed to reveal any clear-cut plant species-specificity clusters within the novel woodland isolates. On examination of the dendrogram three clusters have been formed with 75% homology as a cut-off point. Each of these clusters appear to have a degree of species-specificity, such as cluster 1 containing all of the isolates originating from *Betula pendula* (silver birch) samples and cluster 2 which contained only the *B. graminis* strains (C3AIM, C4DIM and C5AIM). There is also cluster 3 which contained *Quercus* (oak) isolates with at least 80% homology to one another. Another *Quercus* (oak) isolate was linked closely to these isolates with 60% homology the isolates in cluster 3. However, this cluster was not completely representative of all *Quercus* isolates as others could be found elsewhere within the dendrogram.

The combination of the BOX and ERIC fingerprint data has resulted in separating some clusters and distancing some isolates from one another. This event can been seen in the three *B. graminis* strains which are still clustered together by the

relatedness of the DNA profiles is reduced. The combination of the BOX and ERIC DNA fingerprints has also generated fewer clusters at the level of 75% relatedness.

4.10 Amplified fragment length polymorphism (AFLP) analysis of the *Burkholderia* isolates

The BOX and ERIC DNA fingerprint dendrograms failed to generate any comprehensive groupings of the *Burkholderia* species and the novel *Burkholderia* isolates. AFLP has recently emerged as a new method to create taxonomic groupings, it has been reported to be successful in grouping members of the *Burkholderia* genus (Vandamme *et al.*, 2000). This method was used in this study to investigate to what extent this method could group the novel *Burkholderia* isolates according to species specificity or habitat.

The AFLP analysis generated a number of bands from each isolate, dependent on the number of sites recognised by the restriction enzymes (*Eco*RI and *MSe*I). Previous studies have demonstrated that the genome of *Burkholderia* isolates are so varied (Holmes *et al.*, 1998) that the number of bands obtained will be difficult to control. The number of bands generated from this analysis varied from 12 bands to 150 bands.

A selected panel comprising of 79 novel *Burkholderia* isolates and members of the *Burkholderia* genus (obtained form the National Culture Collection of Plant Pathogenic Bacteria (NCPPB) and the Protect library, Central Science Laboratory (CSL), (York) were analysed through AFLP amplification analysis. This analysis revealed some clustering of certain groups of isolates was becoming apparent. However, clusters or groupings specific to key characteristics such as clinical or plant pathogenicity were not produced by this method.

The following AFLP dendrogram (figure 4.10) contains *Burkholderia* isolates (clinical, environmental and novel isolates) and other *Burkholderia* species; it also includes information on where the samples were obtained (for ease the plants were referred to their common names as opposed to their Latin names). The clusters defined within this dendrogram shared 75% homology with each other.
The AFLP dendrogram demonstrated that several clusters could be defined. Cluster 1 contains two *B. graminis* strains and the environmental isolate BCT. Cluster 2, 3, 4

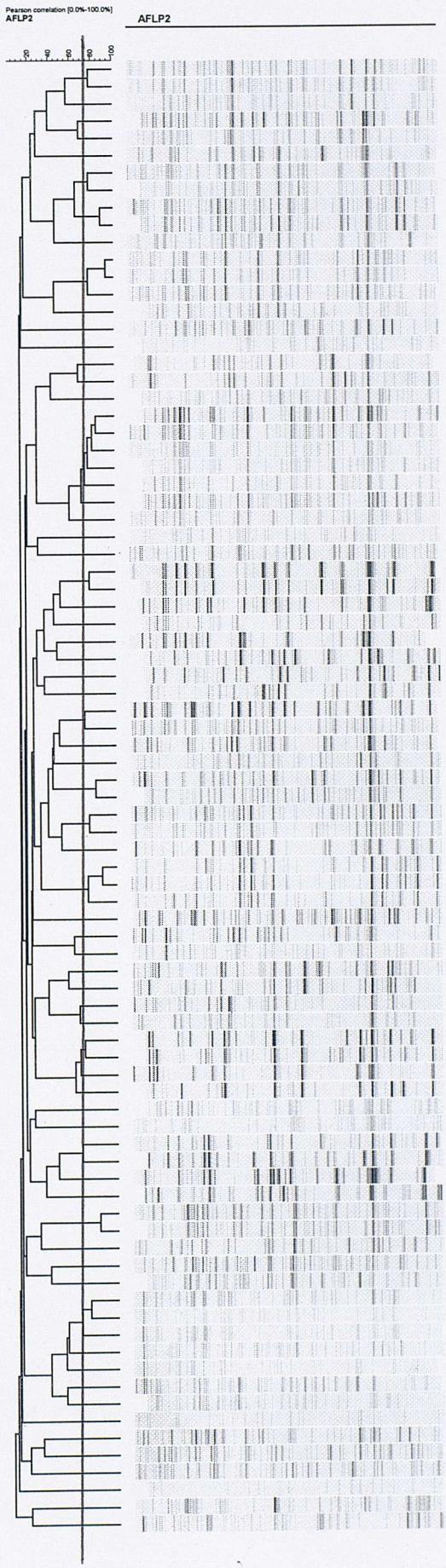
and 7 contained environmental isolates only. These isolates originated from mainly woodland and rhizospheric samples.

Cluster 4 was the only cluster that contained only clinical isolates. However, this cluster was not definitive for all clinical isolates as other clinical isolates could also be found elsewhere on the dendrogram.

None of the clusters formed through this method of analysis generated any definitive habitat or species-specific groupings. However, cluster 1 and 6 revealed a small degree of species specificity containing mushroom and *Allium cepa* (onion) isolates respectively. However, in both cases, other mushroom or *Allium cepa* (onion) isolates could be found elsewhere within the dendrogram. This indicated that these clusters were not definitive in plant species specificity but could benefit from further investigation.

Figure 4.10 Dendrogram of AFLP fingerprints from 72 novel woodland isolates and *Burkholderia* species

The clusters contained within the dendrogram were distinguished at the 75% homology level. Any isolates possessing less that 75% homology were not included in the final analysis of cluster formation and species-specificity.



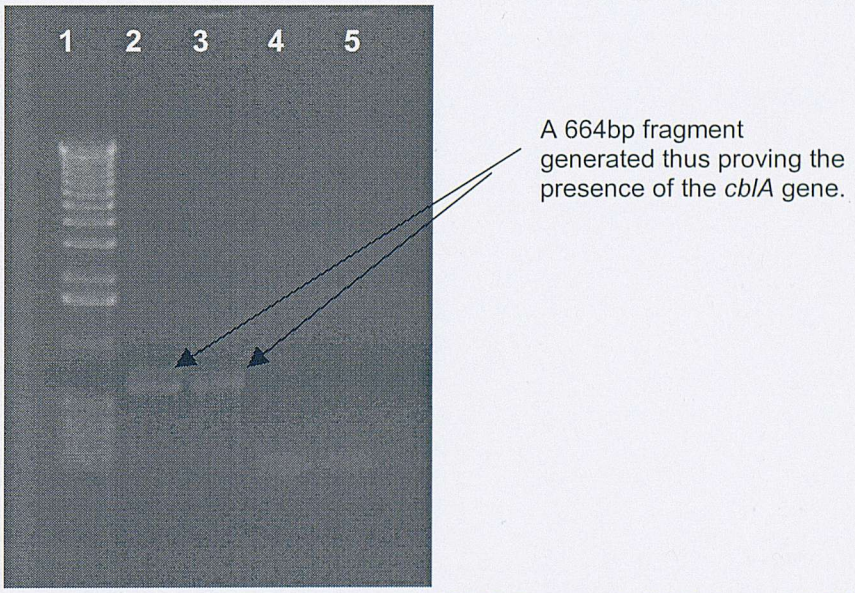
B.	cepacia		P3593	99-11	Soil
B.	cepacia		P3672	OMR16	Roots
B.	cepacia		P3381	WR20B	Woodland roots
B.	cepacia		P3339	WR23S	Roots
B.	cepacia		P3383	WS11	Soil
B.	cepacia			CWP-03	Laural roots
B.	cepacia		P3374	UWE6	Mushroom
B.	cepacia		P3373	UWE2	Mushroom
B.	cepacia		P3346	RW10XB	Rotting wood
B.	cepacia		P3345	RW36	Rotting wood
B.	cepacia			CWR-04	Laural roots
B.	cepacia		P3681	BCT	Prakash
B.	graminis			C3A1M	Maize roots
B.	graminis			C4D1M	Maize roots
B.	cepacia		P3392	ON1	Onion
B.	cepacia		P3671	LS11	Leaf surface- woodland
B.	cepacia		P3375	UWE26	Mushroom
B.	cepacia		P158	NCPPB 3480	
B.	cepacia		P3398	CFPB-2232-94	Clinical
B.	cepacia		P3382	WR21P	Woodland roots
B.	cepacia		P3359	NO14	Environmental
B.	cepacia		P3369	WD21	Woodland
B.	cepacia		P3387	RW24	Rotting wood
B.	cepacia		P3370	WD18	Woodland
B.	cepacia		P3384	WR22P	Woodland roots
B.	cepacia		P3368	WD19	Soil
B.	cepacia		P3385	WS10	Soil
B.	cepacia		P3679	MRL10	Prakash
B.	cepacia			CWS-02	Oak soil
B.	plantarii		P246	ICMP 9426-87	
B.	cepacia		P447	CFBP 1434B	
B.	gladioli	alliicola	P184	794	
B.	cepacia		P451	CFBP 1435	
B.	gladioli	gladioli	P161	NCPPB 1051	
B.	cepacia		P3353	J2552	Botanics
B.	cepacia		P3347	OY715	Onion
B.	cepacia		P3351	J1680	Clinical- CF
B.	cepacia		P3364	C1964	Clinical- CF
B.	cepacia		P3366	WD16	Woodland
B.	cepacia		P3363	J2421	Soil
B.	cepacia		P3400	CFPB-2228-94	Clinical
B.	cepacia		P3352	J1750	Clinical- CF
B.	cepacia		P3399	CFPB-2230-94	Clinical
B.	cepacia		P3592	99-7	Soil
B.	cepacia		P3590	99-10	Soil
B.	cepacia		P3673	ONH	Onion
B.	cepacia		P3591	63-75	Soil
B.	spp.		P3675	RLL7	Leaf litter
B.	cepacia		P3404	SH13E	Woodland
B.	cepacia		P3342	WSP32	Soil
B.	cepacia		P3360	J2861	Soil
B.	cepacia		P3402	CFPB-2233-94	Clinical
B.	cepacia		P3676	BRS55	Birch root soil
B.	cepacia		P3595	98-1	Soil
B.	cepacia		P3391	WR216	Roots
B.	cepacia		P3401	CFPB-2229-94	Clinical
B.	cepacia		P3362	J2315	Clinical- CF
B.	cepacia		P3361	C1576	Clinical- CF
B.	cepacia		P1425	J2394	Clinical
B.	cepacia		P1432	C1524	Clinical
B.	cepacia		P3365	WF23	Agricultural
B.	cepacia			CWR-01	Oak rotting wood
B.	glumae		P935	NCPPB 3673	
B.	glumae		P936	NCPPB 3708	
B.	plantarii		P240	ICMP-9429-87	
B.	gladioli	agaricico.	P409	RR7	
B.	cepacia		P3348	OY716	Onion
B.	cepacia		P3349	OY714	Onion
B.	graminis			C5A1M	Maize roots
B.	cepacia		P3340	WW36	Rotting wood
B.	cepacia		P3390	S512	Agricultural soil
B.	cepacia		P3372	WS8	Sandy soil
B.	cepacia		P3380	WS12	Woodland
B.	cepacia		P3379	WR25B	Woodland
B.	cepacia		P3389	RW4	Pine rotting wood
B.	cepacia		P3377	GR32	Groundsel root
B.	cepacia		P3376	WR24B	Roots
B.	cepacia		P3378	WR25S	Roots
B.	gladioli	alliicola	P1176	NCPPB 3853	
B.	cepacia		P3344	GR36	Roots
B.	cepacia		P3350	OY513	Onion
B.	cepacia		P3393	OY4F	Onion
B.	cepacia		P3396	K14	Potato soil
B.	cepacia		P3386	RW5	Rotting wood
B.	cepacia		P3388	RW26	Pine rotting wood

4.11 Cable A gene PCR amplification

The cable A (*cblA*) gene has been associated with the production of a pilin found in human pathogenic strains of *B. cepacia* such as J2335 which is responsible for the adherence of the bacterial cell to the epithelial lining of the lungs (see section 1.10). To find the *cblA* gene present in any of the novel *Burkholderia* isolates may indicate a potential for human pathogenicity.

The primers used in this PCR were designed by Sajjan *et al.* (1995) to amplify a 664bp fragment of the *cblA* gene. This PCR analysis was used to screen all novel woodland and clinical isolates for the presence of the *cblA* gene. Out of the LMG series of isolates, LMG 18863 and LMG 16656 were both found to contain the *cblA* gene. None of the novel woodland *Burkholderia* isolates obtained from this study were found to contain the *cblA* gene.

Figure 4.11 *CblA* PCR analysis of *B. cepacia* J2315 (LMG 16656) Edinburgh-Toronto epidemic strain and clinical strains of *B. cepacia*



Lane 1 1kb DNA Ladder (Promega), Lane 2 Positive control J2315 *B. cepacia* [GIII], Lane 3 LMG 18863 *B. cepacia* , Lane 4 LMG18821 *B. cepacia*, Lane 5 Negative control.

To ensure that the fragment was indeed the *cblA* gene the fragment was extracted from the gel and ligated into pGEM-T Easy vector, cloned and sequenced. A BLASTX search (Entrez-Nucleotide web-site http://www.ncbi.nlm.nih.gov/) was carried out to

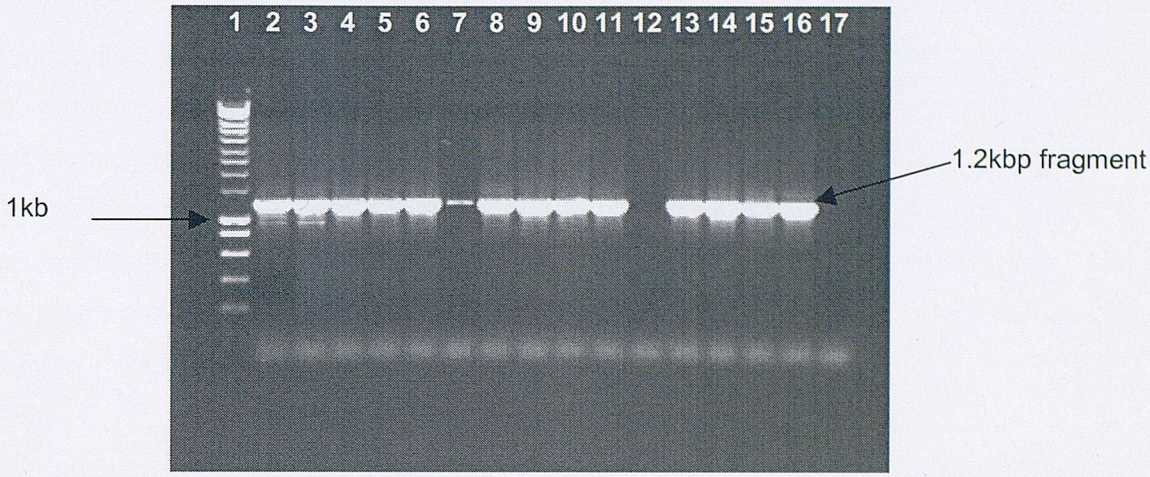
confirm the homology of the sequence obtained to the *cblA* gene previously sequenced (Accession number U10244, Sajjan *et al.*, 1995).

4.12 16S rRNA gene sequencing of novel environmental isolates of *Burkholderia*

The 16S rDNA gene is a highly conserved gene used in determining evolutionary changes between bacteria (see section 1.13). Comparing the 16S rDNA gene sequences of the novel *Burkholderia* isolates with fully characterised species of *Burkholderia* could highlight any habitat or species specific groupings.

From the use of primers at positions 27 and 1240 within the 16S rRNA gene, a 1.2kbp fragment was to be expected from this PCR amplification procedure. For sequencing purposes an internal primer at position 480 within the gene was used to retrieve the complete sequence of the fragment. These sequences were then assembled and the data compiled to check for homology with other known *Burkholderia* 16S rDNA sequences. This was achieved through the use of GCG (Wisconsin package) to assemble and align the sequenced fragments; ClustalX to align the assembled sequences (Thompson *et al.,* 1997) and TreeView to produce a bootstrapped phylogram of all the 16S rRNA sequences (Page, 1996). The 16S sequences were then clipped to all start from the 27f primer site and finish at the 1240 primer site. This in turn would bring all of the sequences statistically closer and more comparable to one another (see figures 4.13, 4.14 and 4.15 for 16S rDNA phylograms).

Figure 4.12 16S rRNA gene product of novel environmental isolates of *Burkholderia*



Lane 1 1kb DNA Ladder (Promega), Lane 2 C3AIM *B. graminis (Graminacae* sp.), Lane 3 CWL-01 *Burkholderia* sp.(*Laurus* sp.), Lane 4 CWP-01 *Burkholderia* sp. (*Laurus* sp.), Lane 5 CWR-01 *Burkholderia* sp. (*Quercus* sp.), Lane 6 CWR-02 *Burkholderia* sp. (*Quercus* sp.), Lane 7 CWS-01 *Burkholderia* sp. (*Quercus* sp.), Lane 8 CWS-02 *Burkholderia* sp. (*Quercus* sp.), Lane 9 CWS-03 *Burkholderia* sp. (*Platanus* sp.), Lane 10 YWS-04 *Burkholderia* sp. (*Ulmus* sp.), Lane 11 YWS-05 *Burkholderia* sp. (*Fraxinus* sp.), Lane 12 YWS-14 *Burkholderia* sp. (*Quercus* sp.), Lane 13 YWS-15 *Burkholderia* sp. (*Quercus* sp.), Lane 14 HWS-05 *Burkholderia* sp. (*Pinus* sp.), Lane 15 HWS-22 *Burkholderia* sp. (*Betula* sp.), Lane 16 HWS-29 *Burkholderia* sp. (*Fagus* sp.), Lane 17 –ve control.

All of the novel isolates were selected for the 16S rDNA PCR analysis. However, only 29 of the novel isolates generated sufficient amounts of DNA for sequencing. When the 16S rDNA PCR amplification was unsuccessful for any of the isolates, the PCR amplification was repeated, in order to attempt to generate the required fragment. The novel isolate in lane 12, YWS-14 (*Quercus* sp.-oak rhizospheric soil) did not produce a 1.2kbp amplicon, but on a subsequent 16S rDNA PCR amplification it generated the required fragment. It was not known why some of the isolates failed to produce the 1.2bp amplicon on one occasion but produces one on another occasion. The isolates which did not generate any product could not be included in this part of the study (the primer sites may have been points of heterogeneity) and further attempts to generate a 16S rDNA fragment was ceased.

4.13 16S rDNA phylogenetic tree

Contained within the following phylograms are 28 novel *Burkholderia* woodland isolates, 3 *B. graminis* strains and a number of other *Burkholderia* species. An out-

group was used to demonstrate the relatedness of the *Burkholderia* species to the novel woodland isolates. The following three phylograms will illustrate the degree of relatedness the novel *Burkholderia* isolates have to each other and fully characterised members of the *Burkholderia* genus. *Staphylococcus aureus* has been used as the out-group to root the phylograms (see figures 4.13, 4.14 and 4.15). The 16S rRNA sequences from Gram negative *Proteobacteriae, Ralstonia euthropha*, *Ralstonia solancearum*, *Ralstonia pickettii* and *Pseudomonas lini* were included in figure 4.15. The addition of these *Proteobacteriacae* sequences helped to demonstrate the evolutionary relationships of the sequences contained within the phylogram (Page & Holmes, 1998).

The addition a Gram positive 16S rDNA sequence from *Staphylococcus aureus* was included as another out-group to further characterise the relationships of all of the sequences contained within this phylogenetic tree. It was hoped that this method could create a grouping which would define the woodland isolates from the *B. cepacia* complex members.

Figure 4.13 16S rDNA phylogenetic tree of novel woodland *Burkholderia* isolates

The following concise phylogram contains the novel woodland isolates of *Burkholderia* and environmental type strains of *Burkholderia*. To create a comparison of how these isolates related within the *Burkholderia* genus some known species were included. The phylogram was rooted with *S. aureus* as the out-group. This phylogram revealed the formation of four clusters, cluster 4 contains woodland rhizospheric isolates and the type strain of *B. fungorum* which was also a rhizospheric isolate. This phylogram shows the formation of 4 main clusters, but none of these clusters have highlighted any habitat or species specificity within them. However, *B. fungorum* can be seen to be close proximity to the woodland isolates, suggesting that the novel isolates contained within clusters 3 and 4 could be related to it.

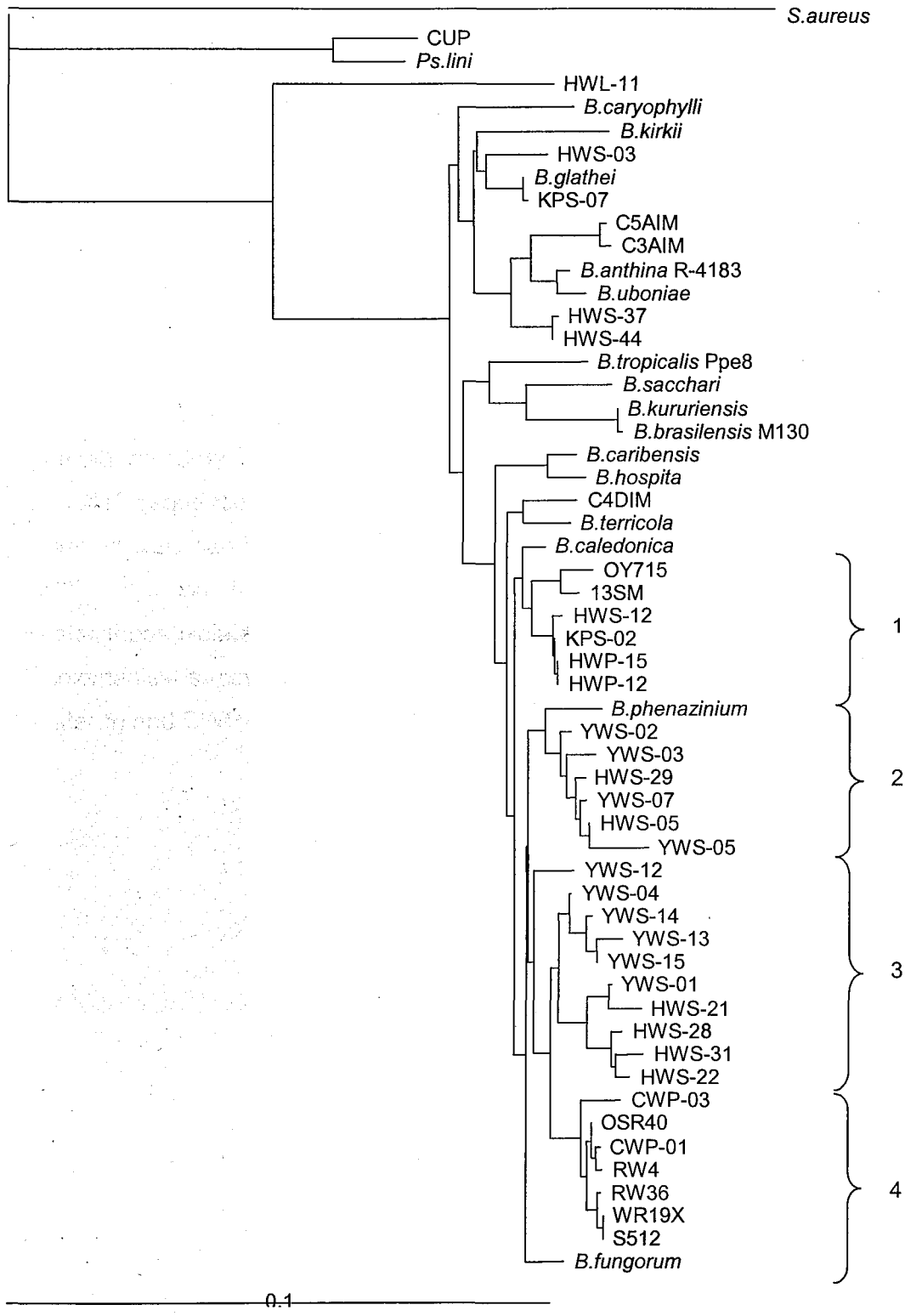
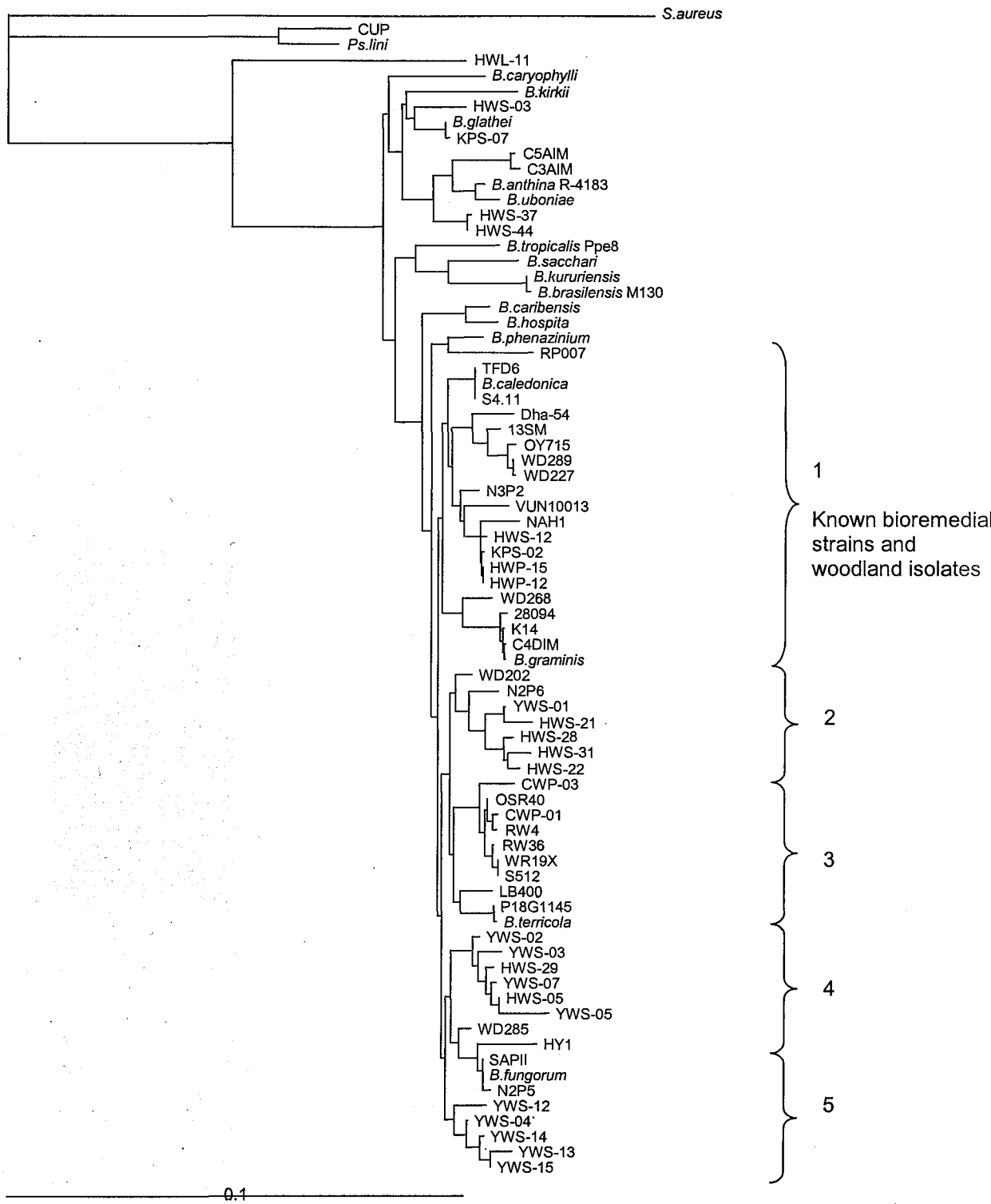

S.aureus
CUP
Ps.lini
HWL-11
B.caryophylli
B.kirkii
HWS-03
B.glathei
KPS-07
C5AIM
C3AIM
B.anthina R-4183
B.uboniae
HWS-37
HWS-44
B.tropicalis Ppe8
B.sacchari
B.kururiensis
B.brasilensis M130
B.caribensis
B.hospita
C4DIM
B.terricola
B.caledonica
OY715
13SM
HWS-12
KPS-02
HWP-15
HWP-12
B.phenazinium
YWS-02
YWS-03
HWS-29
YWS-07
HWS-05
YWS-05
YWS-12
YWS-04
YWS-14
YWS-13
YWS-15
YWS-01
HWS-21
HWS-28
HWS-31
HWS-22
CWP-03
OSR40
CWP-01
RW4
RW36
WR19X
S512
B.fungorum
1
2
3
4
0.1

Figure 4.14 16S rDNA phylogenetic tree of novel woodland isolates and bioremedial *Burkholderia* strains

This phylogram contains the novel woodland isolates of *Burkholderia* and reported bioremediative strains of *Burkholderia*. From this phyiogram the relationship and relatedness of the novel isolates to known bioremediators has been be investigated. It could also help define possible bioremediators from the collection of novel woodland isolates. However, this hypothesis will only be confirmed after further experimental investigation.

To create a comparison to how these novel isolates are related within the genus *Burkholderia* some known species were included. The phylogram was rooted with the 16S rDNA sequence from *S. aureus* as the out-group. The known bioremedial strains of *Burkholderia* can be found within clusters 1, 2 and 3 (*ie.* RP007 Dha-54 S4.11 and N3P2). Also contained within this cluster are novel woodland isolates, in a later chapter these isolates will be tested for any bioremedial properties. The inclusion of the biomedical strains has changed the cluster containing novel isolate *B. fungorum* (cluster 5) and CWP-01 (*Laurus* sp.), which can now be found within cluster 3.


S.aureus
CUP
Ps.lini
HWL-11
B.caryophylli
B.kirkii
HWS-03
B.glathei
KPS-07
C5AIM
C3AIM
B.anthina R-4183
B.uboniae
HWS-37
HWS-44
B.tropicalis Ppe8
B.sacchari
B.kururiensis
B.brasilensis M130
B.caribensis
B.hospita
B.phenazinium
RP007
TFD6
B.caledonica
S4.11
Dha-54
13SM
OY715
WD289
WD227
N3P2
VUN10013
NAH1
HWS-12
KPS-02
HWP-15
HWP-12
WD268
28094
K14
C4DIM
B.graminis
WD202
N2P6
YWS-01
HWS-21
HWS-28
HWS-31
HWS-22
CWP-03
OSR40
CWP-01
RW4
RW36
WR19X
S512
LB400
P18G1145
B.terricola
YWS-02
YWS-03
HWS-29
YWS-07
HWS-05
YWS-05
WD285
HY1
SAPII
B.fungorum
N2P5
YWS-12
YWS-04
YWS-14
YWS-13
YWS-15
0.1
1
Known bioremedial strains and woodland isolates
2
3
4
5

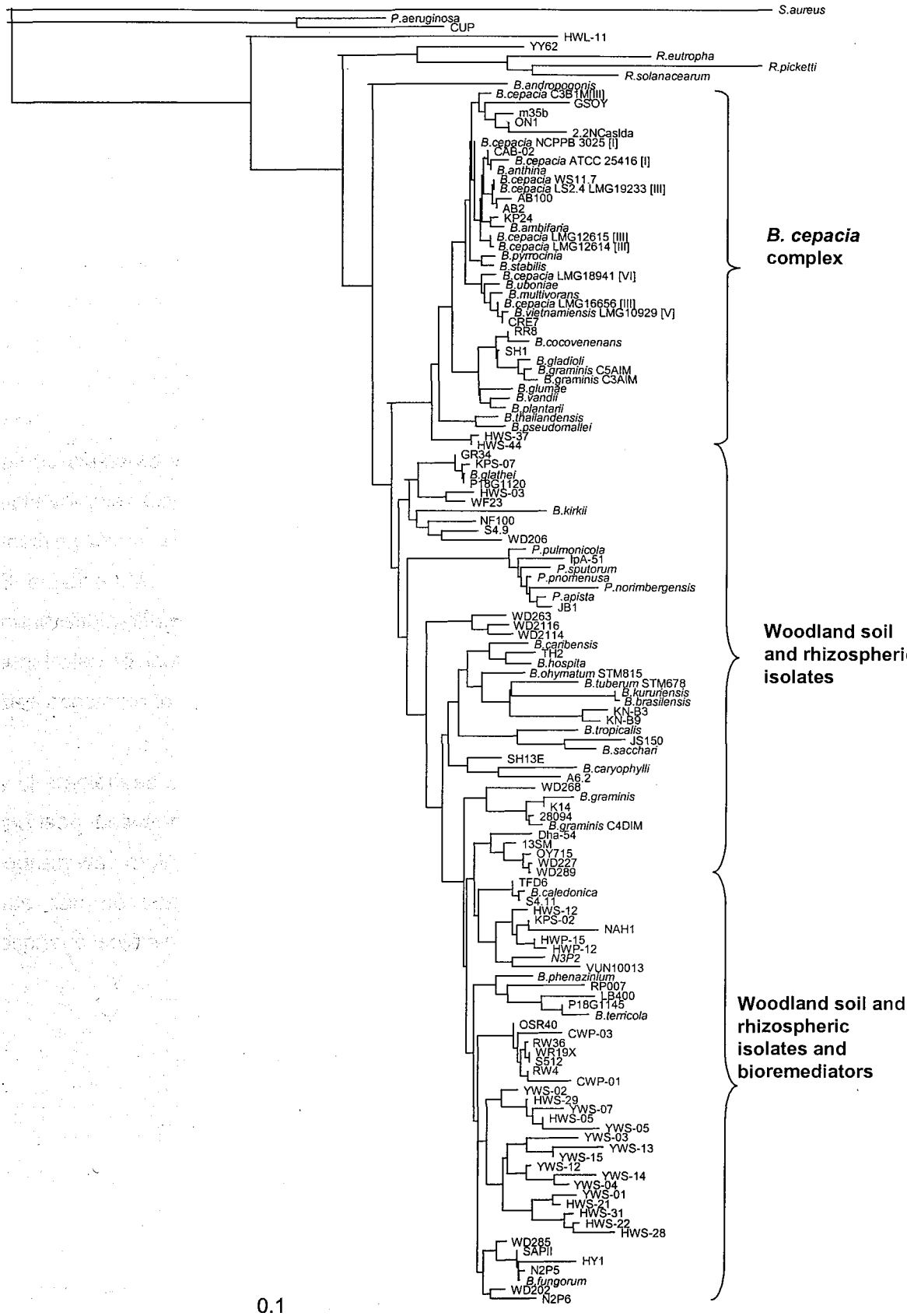

S.aureus
P.aeruginosa
CUP
HWL-11
YY62
R.eutropha
R.picketti
R.solanacearum
B.andropogonis
B.cepacia C3B1[IIIII]
GSOY
m35b
ON1
2.2NCaslda
B.cepacia NCPPB 3025 [I]
CAB-02
B.cepacia ATCC 25416 [I]
B.anthina
B.cepacia WS11.7
B.cepacia LS2.4 LMG19233 [III]
AB100
AB2
KP24
B.ambifaria
B.cepacia LMG12615 [IIII]
B.cepacia LMG12614 [III]
B.pyrrocinia
B.stabilis
B.cepacia LMG18941 [VI]
B.uboniae
B.multivorans
B.cepacia LMG16656 [IIII]
B.vietnamiensis LMG10929 [V]
CRE7
RR8
B.cocovenenans
SH1
B.gladioli
B.graminis C5AIM
B.graminis C3AIM
B.glumae
B.vandii
B.plantarii
B.thailandensis
B.pseudomallei
HWS-37
HWS-44
GR34
KPS-07
B.glathei
P18G1120
HWS-03
WF23
B.kirkii
NF100
S4.9
WD206
P.pulmonicola
IpA-51
P.sputorum
P.phorrhenusa
P.norimbergensis
P.apista
JB1
WD263
WD2116
WD2114
B.caribensis
TH2
B.hospita
B.ohymatum STM815
B.tuberum STM678
B.kururiensis
B.brasilensis
KN-B3
KN-B9
B.tropicalis
JS150
B.sacchari
SH13E
B.caryophylli
A6.2
WD268
B.graminis
K14
28094
B.graminis C4DIM
Dha-54
13SM
OY715
WD227
WD289
TFD6
B.caledonica
S4.11
HWS-12
KPS-02
NAH1
HWP-15
HWP-12
N3P2
VUN10013
B.phenazinium
RP007
LB400
P18G1145
B.terricola
OSR40
CWP-03
RW36
WR19X
S512
RW4
CWP-01
YWS-02
HWS-29
YWS-07
HWS-05
YWS-05
YWS-03
YWS-13
YWS-15
YWS-12
YWS-14
YWS-04
YWS-01
HWS-21
HWS-31
HWS-22
HWS-28
WD285
SAPII
HY1
N2P5
B.fungorum
WD202
N2P6
0.1
B. cepacia complex
Woodland soil and rhizospheric isolates
Woodland soil and rhizospheric isolates and bioremediators

Figure 4.15 16S rDNA phylogenetic tree of novel woodland isolates, *Burkholderia cepacia* complex isolates and strains of *Burkholderia*

This is the definitive *Burkholderia* phylogram containing the novel woodland isolates, *B. cepacia* complex species and other species. This phylogram aimed to illustrate the novel woodland isolates relatedness to other members of the *Burkholderia* genus and the *Proteobactericae* family. The inclusion of the *Proteobactericae* 16S rDNA data revealed how closely related the novel *Burkholderia* isolates were to the fully characterised members of *Burkholderia*. The *Proteobactericae* data revealed that HWL-11 (*Betula* sp.) and CUP (Unknown plant sample) were more related to *Ralstonia* spp. and *Pseudomonas aeruginosa* respectively. The other novel isolates remained clustered with the woodland *Burkholderia* isolates. The members of the *B. cepacia* complex formed a large cluster, but they could not be distinguished into their respective genomovars through the use of this method. Three members of genomovar III (*B. cepacia* LMG 19233, *B. cepacia* LMG12615 and *B. cepacia* LMG 12615) could be found within different sub-clusters within the *B. cepacia* complex cluster. This event demonstrates to inability of the 16S rDNA gene sequence to cluster *Burkholderia* species according to their genomovar status.

Fully characterised *Burkholderia* species were included in this phylogram to enable a comparison between the novel isolates and known species of *Burkholderia*. The phylogram was rooted with *S. aureus* as the out-group. The formation of three distinct groups can be seen within this phylogram, the *B. cepacia* complex, woodland rhizospheric Isolates and the woodland isolates and bioremediators.

4.14 Random fragment length polymorphism (RFLP) of 16S rDNA PCR product from novel *Burkholderia* isolates

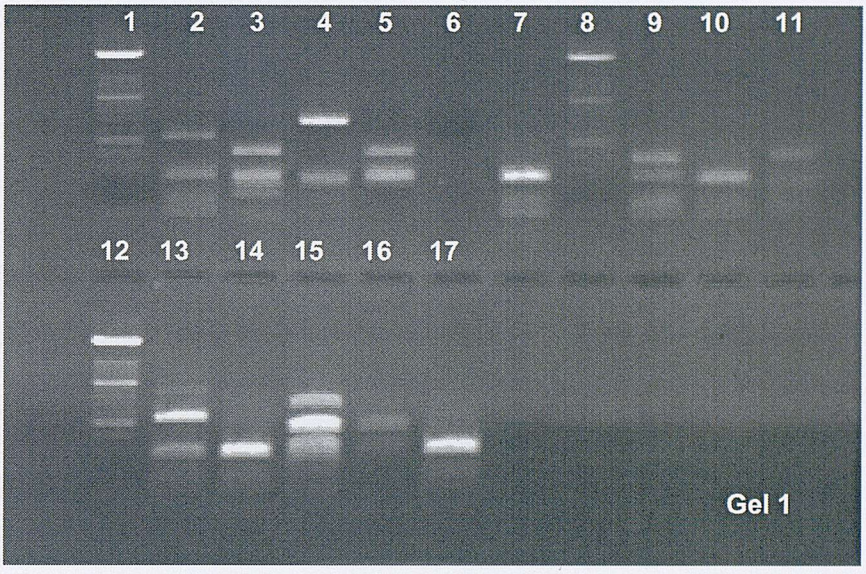
As mentioned in section 4.12, the 16S rDNA gene can be used to measure evolutionary change of micro-organisms. Another method of analysing the relatedness of the 16S rDNA gene is to compare the restriction fragments generated after digestion with restriction enzymes. The more differences in the 16S rDNA gene within these novel isolates, the more variable the RFLP patterns will be. Through comparing the RFLP patterns of the 16S rDNA gene it was hoped that some habitat or species specificity could be detected.

Comparison of the restriction patterns generated from this analysis were made visually. The novel isolates chosen for this experiment were representative isolates from each cluster in the 16S rDNA analysis (see figure 4.13). Through the use of *Dde*I restriction enzyme, 8 different RFLP profiles were generated amongst the novel *Burkholderia* isolates which can be seen on the following gel images.

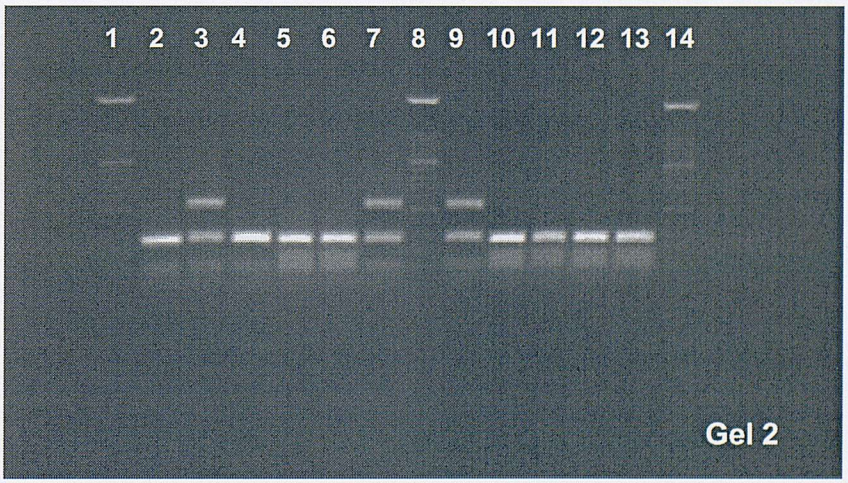
CWP-01 (*Laurus* sp.) and YWS-01 (*Fagus* sp.) were the only two novel *Burkholderia* isolates which had the same RFLP profile. From the following three gel images contained within figure 4.16, there appeared to be no definitive grouping of the novel isolates according to species-specificity or *B. cepacia* genomovar. However, some similarities can be found in some of the RFLP patterns. From Gel 2 it can be seen that the profiles for *B. cepacia* LMG 18821 and *B. cepacia* LMG 18863 could not be distinguished from the *B. stabilis* (genomovar VI) strains; this indicated that this method could not define between species. None of the novel isolates generated a profile that was the same as the onion pathogenic type strain *B. cepacia* 2993. To conclude, this method was unable to distinguish between the plant pathogenic from the clinical or environmental isolates.

Figure 4.16 Random fragment length polymorphism (RFLP) of 16S rDNA PCR product from novel *Burkholderia* isolates

The gel images below contain the RFLP patterns of the 16S rDNA amplicons. The DNA fingerprints generated revealed a number patterns from the various *Burkholderia* species and novel isolates tested.

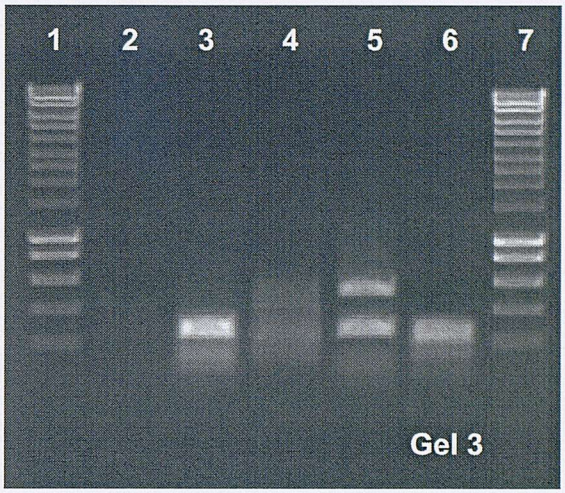


Lane 1 Marker VI (Boeringher Mannheim) 100bp, Lane 2 *B. ambifaria* LMG 19182 [GVII], Lane 3 LMG *B. fungorum* LMG 16225, Lane 4 *B. sacchari* LMG 19450, Lane 5 C3AIM (*Graminacae* sp.), Lane 6 –ve control, Lane 7 CWP-01 (*Laurus* sp.), Lane 8 Marker VI, Lane 9 CWP-02 (*Laurus* sp.), Lane 10 *B. cepacia* J2315 [GIII], Lane 11 KPS-02 (*Quercus* sp.), Lane 12 Marker VI, Lane 13 *B. caledonia* LMG 19076, Lane 14 YWS-03 (*Ulmus* sp.), Lane 15 YWS-04 (*Ulmus* sp.), Lane 15 YWS-07 (*Betula pendula*), Lane 16 *B. cepacia* type strain 2993 [GI].



Lane 1 Marker Hyperladder I (Bioline) Lane 2 *B. vietnamiensis* LMG 10929 [GV], Lane 3 *B. multivorans* LMG 13010 [GII], Lane 4 *B. vietnamiensis* LMG 16232 [GV], Lane 5 *B. cepacia* LMG 16656 [GIII], Lane 6 *B. cepacia* LMG 16659, Lane 7 *B. multivorans* LMG 16660 [GII], Lane 8 Marker, Lane 9 *B. multivorans* LMG 16665 [GII], Lane 10 *B. cepacia* LMG 18821, Lane 11 *B. cepacia* LMG 18863, Lane 12 *B. stabilis* LMG 18870 [GIV], Lane 13 *B. stabilis* LMG 18888 [GIV], Lane 14 Marker Hyperladder.

The three *B. multivorans* isolates (Lanes 3, 7 and 9) all possessed the same RFLP profile none of the novel isolates had. However, the *B. sacchari* RFLP pattern appeared to be similar to the *B. multivorans* pattern. Using 16S rDNA gene RFLP may may be suitable for identifying *B. multivorans* species from other species if modified further.



Lane 1 100bp Marker Boeringer-Mannheim, Lane 2 HWS-03 *Burkholderia* sp. (*Fagus* sp.), Lane 3, *B. cepacia* J2315 [GIII], Lane 4 KPS-02 *Burkholderia* sp. (*Quercus* sp,), Lane 5 KPS-07 *Burkholderia* sp. (*Fagus* sp.), Lane 6 YWS-01 *Burkholderia* sp. (*Fagus* sp.), Lane 7 100bp Marker.

The RFLP profile of *B. cepacia* J2315 appeared to be very similar to the novel isolate YWS-01 (*Fagus* sp.). The two novel isolates (KPS-07 and YWS-01) from *Fagus* sp. (beech) samples appeared to possess different RFLP profiles, thus indicating no species specificity. The lack of distinct RFLP profiles generated from the novel isolates and *Burkholderia* species indicated that this method was not suitable for taxonomic grouping. However, it may prove useful in combination with another method to distinguish between two similar isolates.

4.15 *RecA* gene phylogeny of novel isolates of *Burkholderia*

The *recA* gene is responsible of one of the processes in recombinational DNA repair and homologous genetic recombination. This gene has been used to create the genomovar grouping for a number of the *Burkholderia* species (Coeyne *et al.*, 2001d, Mahenthiralingam *et al.*, 2000a, 200b). The term genomovar is used to describe phenotypically similar but genotypically distinct groups of bacterial strains.

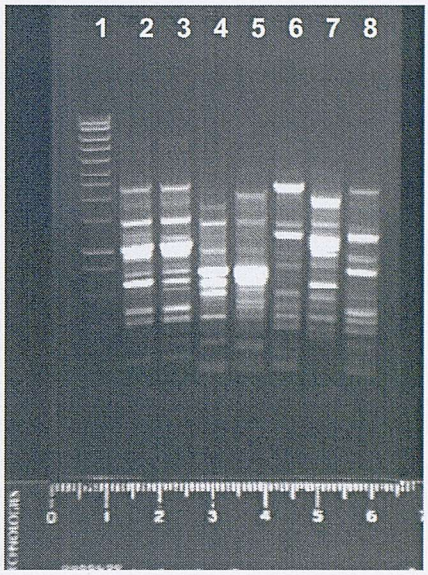
The primers for this analysis were designed from a conserved region of *recA* sequences from other *Burkholderia* spp. and *Proteobacteriacae.* In total, 9 *recA* primers were designed to anneal at specific points and amplify the DNA within the *recA* gene. The various primer combinations (see appendix A10) were used in order to generate a stringent and specific PCR amplification as possible. Primer combination RecA2 and RecA4 proved to be the most successful primer combination in generating fragments which were of *recA* origin. However, the combination of these primers still generated several amplicons following the PCR procedure (see figure 4.17). Therefore, it was necessary to design specific primers that would only amplify a single band of the expected size for the novel isolates, *B. caledonica*, *B. fungorum* and *B. sacchari* strains. The nucleotide and translated peptide sequences were then aligned with known *recA* sequences from various *Burkholderia* species from different genomovars. There were distinct regions of homology within the *recA* sequence of the novel isolates from the different genomovars and within the *B. cepacia* complex. However, there were also subtle nucleotide differences at the 3' terminal region, within these sequences to enable primers to be designed so they could potentially be diagnostic for the novel woodland isolates of *Burkholderia* and the other *Burkholderia* strains.

4.16 *RecA* gene analysis of *Burkholderia* species and novel woodland isolates

Through using the *recA* gene it was hoped that the novel isolates could be allocated to either a pre-existing genomovar or nominated as a new genomovar specific to woodland *Burkholderia* isolates. To obtain *recA* gene data, a set of primers similar to *recA* gene sequences from *Burkholderia* and other members of the family, *Proteobactericae* were designed. Some of the primers contained a 'wobble' base which enabled recognition of two or more bases to allow the primer to anneal. However, using 'wobble' bases within a primer resulted in the reduced specificity of the primer to increase the chances of producing amplicons. The products generated from the PCR analysis were separated by agarose gel electrophoresis (see figure 4.17). The products were then subsequently tested for *recA* gene homology through DNA hybridisation. Any bands showing homology were excised and purified for DNA sequencing.

Figure 4.17 *RecA* gene analysis of *Burkholderia* strains and novel woodland isolates

The primers used in this *recA* PCR analysis were RecA2 and RecA4.



Lane 1 Marker Hyperladder I (Bioline), Lane 2 *B. ambifaria* LMG 19182 [GVII], Lane 3 *B. caledonica* LMG 19076, Lane 4 *B. fungorum* LMG 16225, Lane 5 *B. sacchari* LMG 19450, Lane 6 CWP-01 *Burkholderia* sp. (*Laurus* sp.), Lane 7 YWS-03 *Burkholderia* sp. (*Ulmus* sp.), Lane 8, *B. cepacia* type strain 2993.

*Number in square brackets represents the genomovar status of the species.

The two novel isolates CWP-01 (*Laurus* sp.) and YWS-03 (*Ulmus* sp.) were used as representative strains used to generate *recA* data. The data generated would be then used to investigate the relatedness of these isolates to *B. fungorum*, and whether they could be allocated into any of the existing *Burkholderia* genomovars

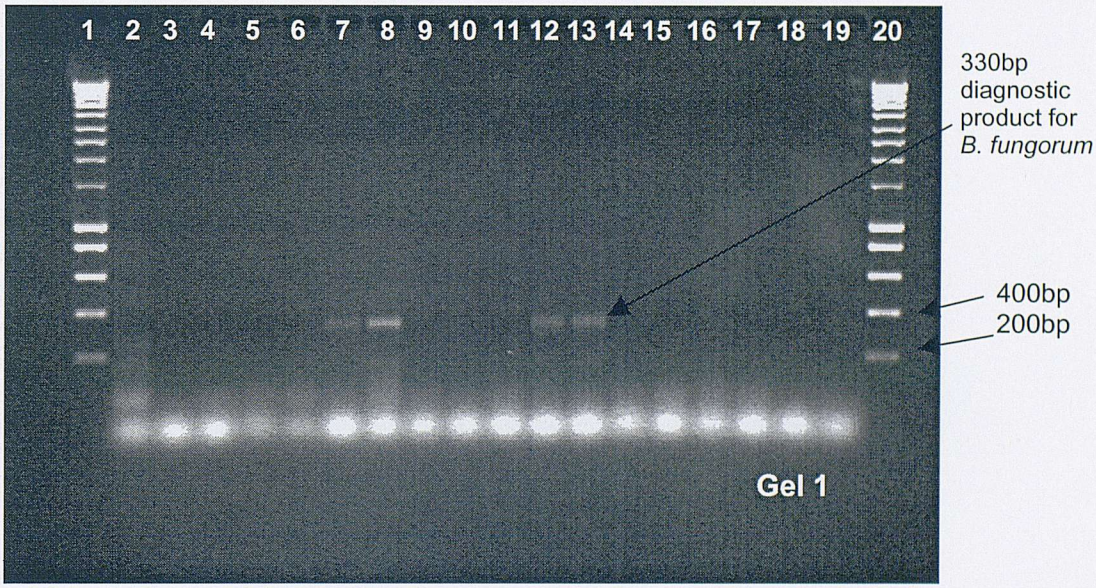
As shown on the gel above the generic *recA* gene primers used in the PCR analysis generated several bands which may have some similarities to the *recA* gene. The gel was transferred onto nitrocellulose membrane and probed against a known *recA* gene product for homology. The *recA* gene sequence generated for the novel *Burkholderia* isolates CWP-01 and YWS-03 were not enough as to identify a nucleotide mis-match to generate diagnostic primers. A length of *recA* sequence containing nucleotide differences was required in order to define the novel isolates from the other *B. cepacia* genomovars.

4.17 Determining *RecA* gene sequence *B. fungorum* and *B. sacchari*

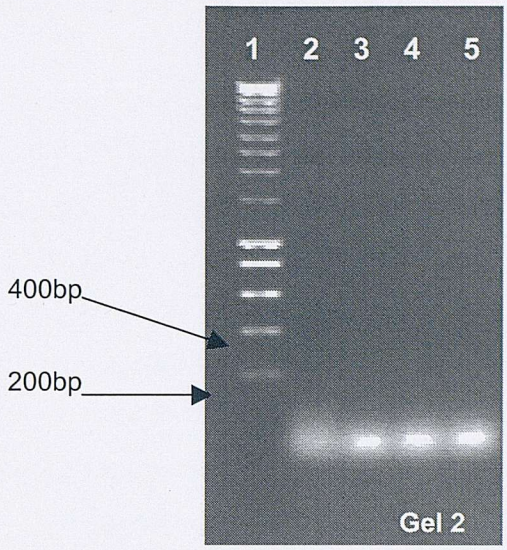
RecA gene sequence and diagnostic *recA* primers were not available for *B. fungorum* and *B. sacchari* at the time of this study. Using the *recA* primers designed for the novel isolates, the PCR products generated from *B. fungorum* and *B. sacchari* were separated and analysed accordingly. Once the sequence data were analysed for these two *Burkholderia* species, diagnostics primers were designed and the extent of species- specificity was checked through PCR analysis (see figure 4.18).

Figure 4.18 *RecA* diagnostic primers for *B. fungorum.*

To test the sensitivity and specificity of the *B. fungorum* diagnostic primers a panel of *B. cepacia* complex species and 4 strains of *B. fungorum* were included in the PCR analysis. The *recA* primers used in this analysis were FunF and FunR.



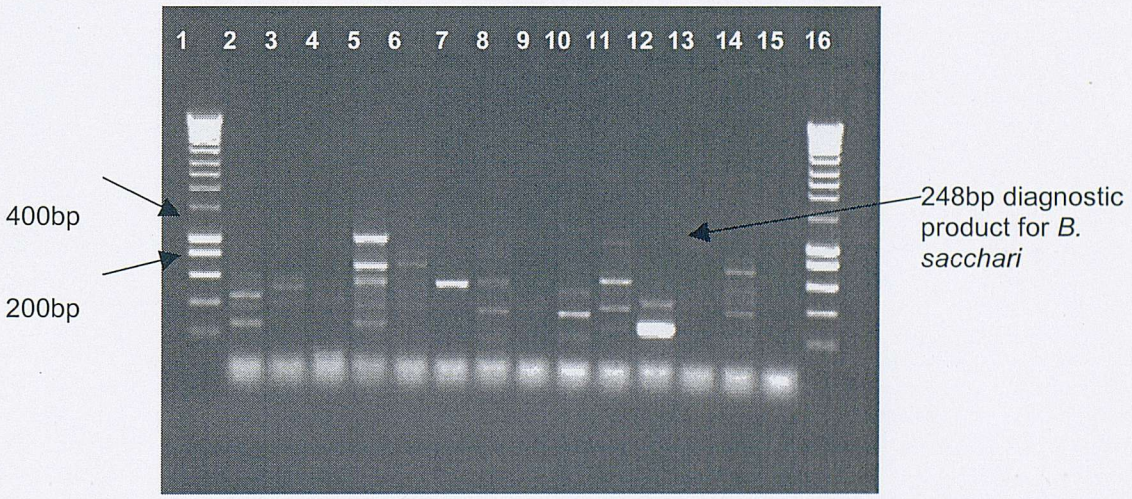
Lane 1 Marker Hyperladder I (Bioline) Lane 2 CWP-01 *Burkholderia* sp. (*Laurus* sp.), Lane 3 YWS-03 *Burkholderia* sp. (*Ulmus* sp.), Lane 4 *B. cepacia* type strain 2993 [GI], Lane 5 *B. vietnamiensis* LMG 10929 [GV], Lane 6 *B. pyrrocinia* LMG 14191 [GIX], Lane 7 *B. fungorum* LMG 16225, Lane 8 *B. fungorum* LMG 16307, Lane 9 *B. cepacia* LMG 16654 [GIII], Lane 10 *B. cepacia* LMG 16656 [GIII], Lane 11 *B. multivorans* LMG 16660 [GII], Lane 12 *B. fungorum* LMG 18809, Lane 13 *B. fungorum* LMG 18810, Lane 14 *B. vietnamiensis* LMG 18836 [GV], Lane 15 *B. cepacia* LMG 18941 [GVI], Lane 16 *B. caledonica* LMG 19076, Lane 17 *B. ambifaria* LMG 19182, Lane 18 *B. sacchari* LMG 19450, Lane 19 *B. fungorum* LMG 20227, Lane 20 Marker Hyperladder I.



Lane 1 Marker Hyperladder I (Bioline) Lane 2 *B. anthina* LMG 20983 [GVIII], Lane 3 *B. terricola* LMG 20594, Lane 4 *B. hospita* LMG 20598.

Figure 4.19 *RecA* diagnostic PCR for *B. sacchari.*

Through sequencing the *recA* gene of using generic *recA* primers, specific primers were designed on the *recA* sequence unique to *B. sacchari*. Diagnostic primers were designed (SacF and SacR), the efficacy of the primers can be seen on the agarose gel below.



Lane 1 Marker Hyperladder I (Bioline), Lane 2 *B. cepacia* type strain 2993 [GI], Lane 3 *B. vietnamiensis* LMG 10929 [GV], Lane 4 *B. fungorum* LMG 16225, Lane 5 *B. cepacia* LMG 16654 [GIII], Lane 6 *B. cepacia* LMG 16656 [GIII], Lane 7 *B. multivorans* LMG 16660 [GII], Lane 8 *B. vietnamiensis* LMG 18836 [GV], Lane 9 *B. cepacia* LMG 18863 [GIII], Lane 10 *B. caledonica* LMG 19076, Lane 11 *B. ambifaria* LMG 19182 [VII], Lane 12 *B. sacchari* LMG 19450, Lane 13 CWP-01 *Burkholderia* sp. (*Laurus* sp.), Lane 14 YWS-03 *Burkholderia* sp.(*Ulmus* sp.), Lane 15 negative control, Lane 16 Marker Hyperladder I.

**The numbers in the square brackets represent the genomovar status of the species.*

4.18 *RecA* species specificity for *B. fungorum* and *B. sacchari*

The findings from this study revealed that the *recA* sequences obtained from the novel woodland isolates were not sufficient in providing sequence data for the design of a species-specific primer combination. However, the *recA* primers designed for the rhizospheric *B. fungorum* (see figure 4.18) and *B. sacchari* (see figure 4.19) species both generate diagnostic species- specific amplicons. The size of the diagnostic PCR-amplicon was approximately 330bp for *B. fungorum* and this was confirmed in all but one of the strains used in this analysis. The amplicon generated by the *B. sacchari* LMG 19450 was sized at 248bp. The *B. sacchari* primers also generated the 248 bp amplicon with *B. cepacia* type strain 2993, *B. multivorans* and *B. ambifaria*. However, the 248bp amplicon was more intense with the *B. sacchari* DNA than the other species (see figure 4.19). It was not possible to obtain sufficient *recA* gene sequence data for *B. caledonica* to design species specific primers.



Figure 4.20 Translated *recA* gene sequence from novel isolates, *B. fungorum*, *B. sacchari* and *B. caledonica*



4.19 *RecA* phylogeny of *Burkholderia* species and novel *Burkholderia* isolates

Generating *recA* gene data appeared to be more successful for *B. fungorum* than for *B. sacchari*. Only two novel isolates were selected for this section of the study due to their relatedness to *B. fungorum*. YWS-03 (*Ulmus* sp.) can be found clustered closely to *B. fungorum* and CWP-01 (*Laurus* sp.) was clustered separately from these two. These novel isolates were selected to determine whether there would be a similarity in the *recA* sequence of these three isolates or whether YWS-03 would be closer to *B. fungorum* than CWP-01.

From the following *recA* phylograms (see figures 4.21 and 4.22) it was evident that the *recA* gene sequence data from novel isolates CWP-01 and YWS-03 may be closely related to strains in genomovar III.

Figure 4.21 *RecA* gene phylogeny of *Burkholderia* species and novel *Burkholderia* isolates – full sequence (and genomovar status)

The *recA* gene sequence of *B. fungorum*, *B. sacchari*, CWP-01 (*Laurus* sp.) and YWS-01 (*Ulmus* sp.) where aligned with the sequences of *Burkholderia* spp. contained in other genomovars to create a phylogram. The *recA* gene sequence of *B. fungorum* was not as closely related to YWS-03 as first anticipated by reference of the 16S rDNA phylogram. The *recA* phylogram demonstrated that novel isolates CWP-01 and YWS-03 were closely related to the members of genomovar IIIa.

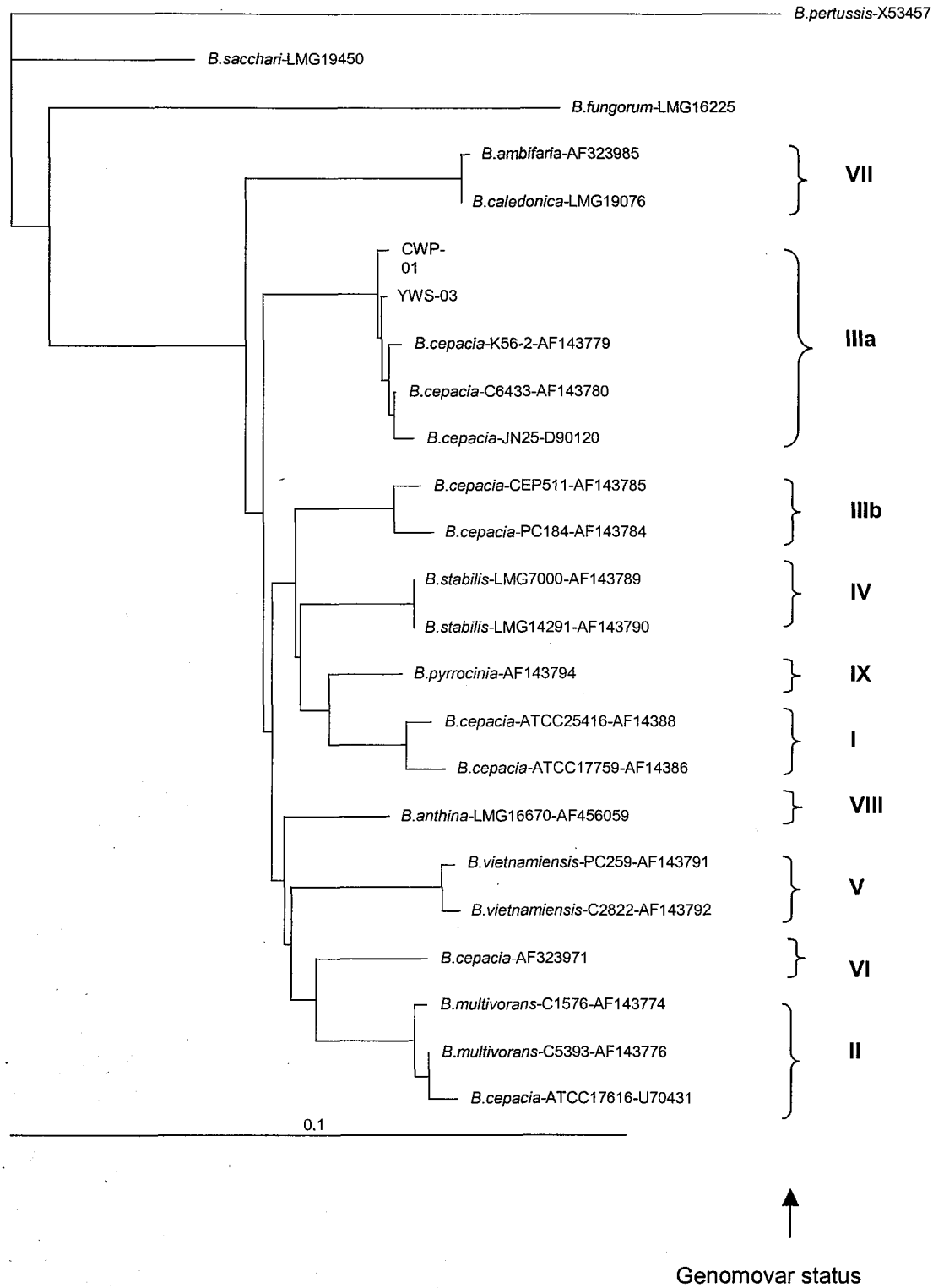
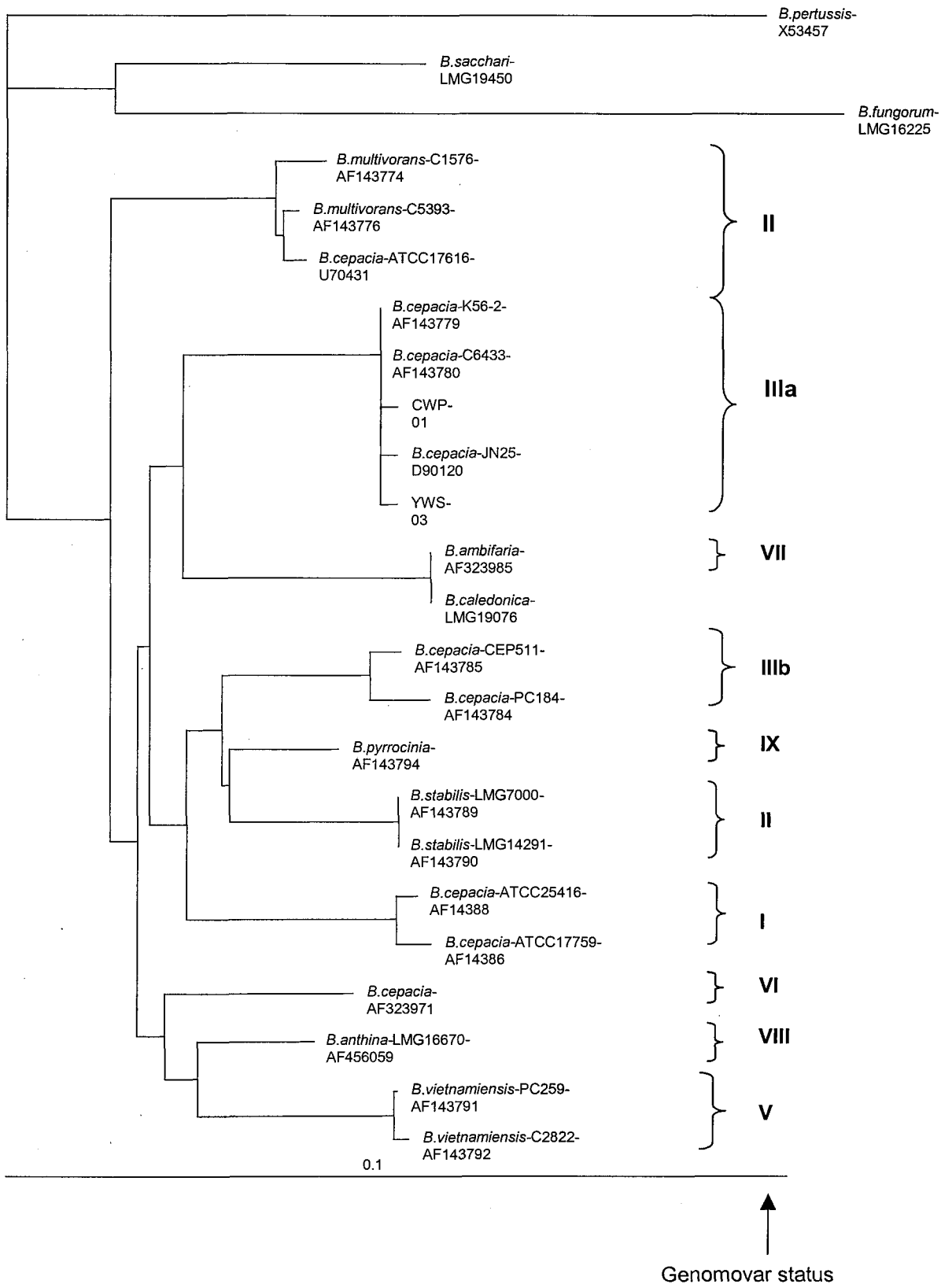

B.pertussis-X53457
B.sacchari-LMG19450
B.fungorum-LMG16225
B.ambifaria-AF323985
B.caledonica-LMG19076
VII
CWP-01
YWS-03
B.cepacia-K56-2-AF143779
B.cepacia-C6433-AF143780
B.cepacia-JN25-D90120
IIIa
B.cepacia-CEP511-AF143785
B.cepacia-PC184-AF143784
IIIb
B.stabilis-LMG7000-AF143789
B.stabilis-LMG14291-AF143790
IV
B.pyrrocinia-AF143794
IX
B.cepacia-ATCC25416-AF14388
B.cepacia-ATCC17759-AF14386
I
B.anthina-LMG16670-AF456059
VIII
B.vietnamiensis-PC259-AF143791
B.vietnamiensis-C2822-AF143792
V
B.cepacia-AF323971
VI
B.multivorans-C1576-AF143774
B.multivorans-C5393-AF143776
B.cepacia-ATCC17616-U70431
II
0.1
Genomovar status

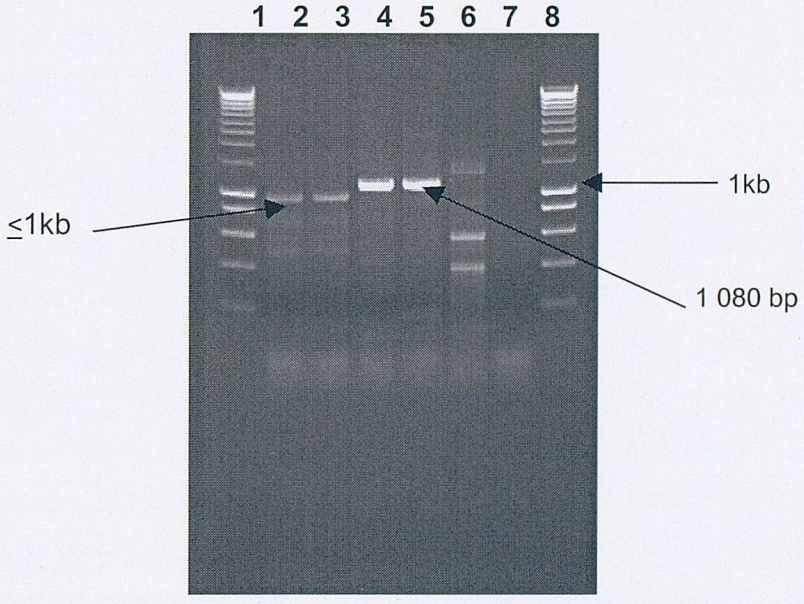
Figure 4.22 *RecA* gene sequence phylogram of *Burkholderia* isolates- Clipped sequence 800bp length (and genomovar status)



4.20 Presence of the *nifK* gene in novel woodland rhizospheric *Burkholderia* isolates

The *nif* group of genes are responsible for the fixation of nitrogen, *nifK* is one of the many genes within the *nif* operon. The presence of a *nif* gene may indicate the ability of the isolate to fix nitrogen. Recently, it has been shown that nitrogen fixation has been found in some *Burkholderia* spp.; investigating the presence of the *nifK* gene may reveal this property in some of the novel isolates. Primer combinations *Nif*KF and *Nif*KR (Minerdi *et al.*, 2001) were used to investigate the potential presence of the nitrogen fixation (*nif*) genes within the novel woodland isolates of *Burkholderia*.

Figure 4.23 *NifK* gene amplification of novel isolates and *B. vietnamiensis*.



Lane 1 Marker Hyperladder I (Bioline), Lane 2 CWP-01 *Burkholderia* sp. (*Laurus* sp.), Lane 3 *B. cepacia* type strain 2993, Lane 4 *B. vietnamiensis* LMG 10929, Lane 5 *B. vietnamiensis* LMG 16232, Lane 6 *Klebsiella pneumoniae* containing a *nifK* construct*, Lane 7 negative control, Lane 8 Marker Hyperladder I.

*This culture was kindly donated by Wendy Cannon of Professor Martin Buck's Laboratory, Imperial College London.

The novel *Burkholderia* isolates were selected clustered closely with the nitrogen fixing *Burkholderia* isolates in the 16S rDNA phylogram. Out of the 20 representative woodland isolates tested, only CWP-01 (*Laurus* sp.) generated an amplicon similar in size to the *nifK* fragment generated by the positive control strains (see figure 4.23). The novel woodland isolate, CWP-01 and *B. cepacia* type strain generated several fragments from this PCR amplification. The amplicons generated from novel isolates

CWP-01 and *B. cepacia* type strain 2993 appeared to be slightly smaller than the fragment generated from the *B. vietnamiensis* strains. When these fragments were hybridised with the *nifK* gene from *B. vietnamiensis* LMG 10929, there was no apparent homology under strict hybridisation conditions.

4.21 DNA-DNA binding of whole genomic DNA of *Burkholderia* species and novel woodland species

Genomic profiling has been carried out on *Burkholderia* species and the novel *Burkholderia* isolates from whole genome profiling to gene sequencing. None of these methods has successfully created a definitive grouping of the novel isolates and the *Burkholderia* species. To confirm or reject the small groupings created through these various methods, DNA-DNA hybridisation can be used to determine the level of relatedness.

By measuring the kinetics of single stranded DNA binding onto another single stranded DNA, it is hoped that relative homology of the two DNA strands can be determined. The more homologous the DNA strands are to each other the faster and stronger they will bind to each other, resulting in a high fluorescence reading. The following table (see table 4.1) shows the varying binding values of the novels isolates, *B. cepacia* 2993, *B. caledonica*, *B. fungorum* and *B. sacchari.* The level of species identification is with a $\geq$70% binding value, using this value it could be seen that RW36 appear to be similar to *B. sacchari* (69-77%), YWS-01 (*Fagus* sp.-beech) (15-76%) and YWS-03 (*Ulmus* sp.-elm) (26-71%). However, the variability of the binding values for YWS-01 and YWS-03 are a cause for concern. *Fraxinus* sp. (ash) isolate, YWS-05 also showed a high binding value with another novel *Fagus* sp. isolate YWS-01 (28-73%).

The DNA binding values were highly variable between the novel isolates and the four *Burkholderia* species used. The variability could be due to the labelling process of the probe. Any free photobiotin was removed but unlabelled DNA was not removed. This may have resulted in unlabelled DNA competitively binding onto the test (bound) DNA, giving a lower second reading.

Whole genomic DNA binding showed did not reveal plant species specificity due to the lack of grouping of the novel isolates. The results of the DNA binding also show that none of the novel isolates were related closely to any of the *Burkholderia* species used in this experiment.

Table 4.1 Binding values of whole genomic DNA from *Burkholderia* species and novel woodland isolates- Microtitre format

The relative homology of the whole genomic DNA was represented by the binding kinetics of the bound and probe DNAs over a period of 2h. Each strain or isolate was repeated in triplicate, an average of the binding values was calculated and used for this table.

BOUND DNA →

PROBE DNA ↓

	2993	CAL	FUN	SAC	HWS-03	OY715	RW36	YWS-01	YWS-03	YWS-05
***B. cepacia* 2993**	**100**	31	15	12	23	34	61	15	31	16
B. caledonica	24	**100**	13	9	9	53	47	43	48	43
B. fungorum	4	1	**100**	44	7	0	37	34	2	28
B. sacchari	53	15	9	**100**	27	13	77	14	23	14
HWS-03	12	3	33	12	**100**	1	57	22	7	43
OY715	9	6	14	26	52	**100**	49	5	17	6
RW36	69	22	36	46	39	43	**100**	15	26	21
YWS-01	29	4	25	9	8	7	76	**100**	10	73
YWS-03	21	23	43	27	8	48	71	13	**100**	13
YWS-05	16	34	34	16	41	64	23	28	32	**100**

B. cepacia type strain 2993 displayed relatively low binding values against the panel of DNAs tested. This results show that none of the novel isolates were related to the type strains used in this study. The DNAs of the two onion isolates (*B. cepacia* 2993 and OY715) showed little relation to each other with binding values only as high as 34%. This study showed that the DNA of *Burkholderia* spp. used were so different to one another that is appears that the novel isolates were not as related to the type strains used as the 16S rRNA sequence data suggested.

4.22 DISCUSSION OF GENOTYPIC DATA

As mentioned at the start of this chapter the following hypotheses will be addressed:

- Common genotypic characteristics are present in *Burkholderia* isolates found in woodland habitats
- Novel woodland *Burkholderia* isolates be clustered according to species of tree or plant.
- Through polyphasic analysis, the clinical, plant pathogenic, environmental and bioremedial isolates be clustered within their respective groups.
- A new *Burkholderia* species be created through 16S DNA analysis of woodland isolates with other *Burkholderia* isolates and defined *Burkholderia* species.

These hypotheses will be either upheld or rejected in the following discussion.

The use of molecular methods has enable the species within the genus *Burkholderia* to be distinguished. The use of random repetitive regions such as BOX element and ERIC within genomic DNA has proved itself to be a powerful tool in partially grouping the *Burkholderia* genus. (Coenye *et al.*, 2000a). However, it failed to group all of the *Burkholderia* species into habitat specific clusters and it also did not allow for characterisation of human or plant pathogenicity. It did enable a certain clustering of the novel woodland isolates which opened up other avenues of investigation into the molecular dynamics of these novel isolates. The effects of habitat, plant species specificity on the molecular characteristics of *Burkholderia* isolates was investigated during the 3 year study.

The use of BOX (see figure 4.5) and ERIC (see figure 4.8) amplification did not reveal any clustering in relation to the habitat or species specificity; this was the same finding when these two analyses were combined for a potentially higher level of discrimination. Some degree of species-specificity regarding tree species from which the isolate was obtained was seen in a small cluster formation containing the novel isolates from *Quercus* sp. (oak) HWS-44, P13F and HWS-37. Just outside the 75% correlation limit, another *Quercus* sp. isolate CWS-01 could be found adjacent to these isolates. It would be a large assumption to say that this cluster was specific for *Quercus* sp. (oak) isolates, as other *Quercus* sp. isolates (CWR-01, CWR-02 and CWS-02) could be found scattered throughout the dendrograms. These *Quercus* sp. isolates were also from the same woodland sites. This suggests that the oak

specificity should be investigated further in future studies to confirm whether there is any species specificity with these isolates. The grouping of members from a genus into geographically specific groups had also proved to be difficult in another genus, *Clavibacter* (Smith *et al.*, 2001).

The DNA fingerprint data from the *Burkholderia* studies carried out by Jane Richardson were combined with the novel woodland data. Unfortunately, the internal controls revealed that the fingerprints generated by the two operators differed from each other. Therefore, the combined data could not be utilised to investigate the evolution or relatedness of the novel woodland isolates compared to the fully characterised and establish species within the genus *Burkholderia*. However, the combination of the two data sets, demonstrated that the novel woodland isolates still clustered together with those from Jane Richardson's collection. However, the exact relatedness of these woodland isolates were not known or would not be known until all of the isolates in the combined BOX and ERIC PCR analyses are repeated by the same operator at the same time. To repeat the BOX and ERIC fingerprinting of Jane Richardsons' collection with the novel *Burkholderia* isolates and *Burkholderia* species will provide clearer picture of how all of the woodland isolates relate to the other members of the genus *Burkholderia*.

The inability to combine the two data sets suggested that although BOX and ERIC are robust molecular methods in bacterial taxonomy, variations in fingerprint generation was evident between different operators and time points (figure 4.3 and figure 4.4). As previously mentioned by Jane Richardson's studies, the use of older cultures or cultures which have been continuously sub-cultured resulted in the generation of fingerprints which varied from one to another. These differences are small but are enough as to affect the relatedness of the isolates contained within the dendrogram.

4.22.1 AFLP

The use of AFLP analysis (figure 4.10) to confirm previous taxonomic groupings has been shown to be reliable for the genus *Burkholderia* (Coenye *et al.*, 1999b; 2001d). The AFLP results of the *Burkholderia spp.* revealed that the number of bands generated varied greatly from 12 to over 150 bands. Due to this large variation there appeared to be a high level of discrimination of the isolates to a point that generating common banding and clustering proved to be difficult. The alterations made by increasing primer combinations did not seem to reduce or regulate the band numbers

within a fingerprint profile. The number of bands may be reduced through the omission of the preselective PCR stage. Attempts were made to select a set range of bands for this analysis to reduce the specificity level. This was achieved through selecting bands between 100-300bp in size. This resulted in some cluster formation. However, it remained unsuccessful in categorising the isolates into genomovars or into isolates possessing key characteristics. There are some habitat-specific clusters within this dendrogram for example a woodland cluster (cluster numbers 1, 2, 3 and 5) appeared to only contain woodland and rhizospheric samples. Unfortunately, no further evidence was available to indicate plant species-specificity for the members of this cluster. There appeared to be a group of clinical isolates in cluster number 4, though the specificity of this group was not confirmed due to the presence of other clinical isolates elsewhere within the dendrogram.

AFLP is a powerful tool which is capable of separating organisms of the same species to the point where they could appear to be different species (personal observation). The band numbers were adjusted through selecting certain band sizes, this created a better cluster formation within the AFLP dendrogram. However, the band size range used did not generate any specific grouping, by selecting other band sizes it could produce a species-specific grouping. Alongside to this, the omission of preselective PCR during AFLP or the use of different primers could produce a better clustering of the novel and isolates. The AFLP technique proved itself to be too discriminating to be able to relate the novel species to known members of the *Burkholderia* genus. This could also be due to the large genome same, genetic diversity within the genus and the genomic plasticity known to *Burkholderia* species.

It is important to compile a taxonomic grouping to reduce the risk of introducing strains of *Burkholderia* into the environment which may have the potential to be pathogens in humans or in onion crops. Extensive analysis must be carried out and the AFLP process optimised before any final conclusions are to be made.

4.22.2 Presence of the *cblA* gene

In a previous study by Richardson *et al*, (2001) only one non-CF isolate of *B. cepacia* was found to possess the *cblA* gene. This may have demonstrated the possibility of gene transfer between two types of clinical isolates. The *cblA* gene is one of the virulence factors used in attaching the bacterium onto the CF lung epithelial cells (Sajjan *et al.*, 1995; Sun *et al.*, 1995; Pitt *et al.*, 1996; Sajjan *et al.*, 2002). Presence of

this gene does not predetermine the isolates ability to become a virulent pathogen, it merely demonstrates a potential.

The screening for the *cblA* gene in environmental isolates is necessary in order to assess the risk of *Burkholderia* usage for biocontrol and bioremediation purposes. To determine the origins of the *cblA* gene, it was hoped to reveal whether the presence of this gene within an environmental isolate was stable; or whether the insertion of a pathogenicity island was temporary and unstable. It was demonstrated that one environmental isolate, NCPPB 3025 (an *Allium cepa* (onion) pathogenic strain) contained the *cblA* gene (Richardson *et al.,* 2001). The presence of this gene in an environmental / non-human pathogenic isolate is a cause for concern should this bacterium be widely used in biocontrol and bioremediation. The continual screening for the *cblA* gene and other human pathogenicity / virulence genes (such as type III secretion systems) is essential in the interest of human health. However, it should not be forgotten that the *cblA* gene is not the only indicative marker for human pathogenicity of the *Burkholderia* isolates. Pathogenicity and epidemic markers such as the *esmR* (Clode *et al.*, 2000), BCESM (Mahenthiralingam *et al.*, 1997) and the presence of a type III secretion system (Winstanley *et al.*, 1999; Winstanley & Hart 2001; Rainbow *et al.*, 2002) must also be taken into account when evaluating potential human pathogenicity.

4.22.3 16S rDNA sequencing

The 16S rDNA data generated an interesting aspect in the novel woodland *Burkholderia* story. Distinct clustering was evident within the *Burkholderia cepacia* complex, from other *Burkholderia* species and novel isolates. Clustering was also found in the characterisation of bioremedial strains of *Burkholderia* (Fain & Haddock, 2001). The 16S rDNA phylogram could literally be split into three parts (excluding the outgroup cluster), *B. cepacia* complex, bioremediators, and novel woodland and rhizospheric isolates. Some of the novel isolates were found to be closely related to bioremediators and these isolates were tested for bioremedial properties, to be discussed in a later chapter. In the meantime, none of the novel woodland isolates could be found contained within the *B. cepacia* complex cluster. This demonstrated that the 16S rDNA sequence data was effective in separating the woodland / environmental isolates from the strains known to cause human and plant pathogenicity (*i.e.*, the *B. cepacia* complex).

The woodland soil and woodland rhizospheric cluster demonstrated that some of the novel isolates were closely related to other rhizospheric endophytic strains of *Burkholderia*. From example, *Burkholderia* strains N2P5, SAPII and WD285 appear to be closely related to the fungal rhizospheric species, *B. fungorum*. A cluster containing the isolates HWS-12 (*Betula* sp.), KPS-02 (*Quercus* sp.), HWP-12 (*Fagus* sp. were all in close proximity to the rhizospheric species *B. caledonica*. There are other woodland clusters formed from this analysis containing the novel isolates and other environmental isolates, thus possibly indicating a new species. Species-specific characterisation of the novel woodland isolates was not evident from this form of analysis.

To analyse the relatedness of the novel isolates to known environmental species of *Burkholderia*, excess sequence data from other species were removed to produce a 'concise' *Burkholderia* phylogram (see figure 4.13). This 'concise' phylogram enabled the relatedness of the novel isolates with other *Burkholderia* species to be visualised. Within this phylogram, 4 clusters could be defined, it showed that several novel isolates were closely related to the some fully characterised species of *Burkholderia*. It appeared that several of the novel woodland isolates are closely related to *B. fungorum*, a fungal isolate (cluster 4). On closer inspection it can be seen that CWP-01 was contained within a cluster harbouring the *B. fungorum* strain (see figure 4.13). Cluster numbers 2, 3 and 4 appear to form separate woodland clusters with cluster 2 being in close relation to *B. phenazinium*. The isolates contained within cluster 4 appeared to have a close relation to *B. fungorum*. Within this cluster there was no habitat or plant specificity between the isolates.

In previous papers the use of random fragment length polymorphism (RFLP) has helped in further differentiating between the members of a cluster (Segonds *et al.*, 1999; Magalhães Cruz *et al.*, 2001). For example, YWS-04 (*Ulmus* sp.) and YWS-07 (*Betula* sp.) were both within close proximity to each other on the 16S rDNA phylogram, but they could be distinguished further through their different RFLP patterns (see figure 4.16, lane 15 and lane 16 on RFLP Gel1).

4.22.4 *RecA* gene sequencing

The *recA* gene has been used extensively in the classification of the *Burkholderia* species into genomovars contained within the *B. cepacia* complex. Each *Burkholderia*

species possess distinct properties and the *recA* sequence phylogeny has been successful in grouping the different species within the genus into key genomovars.

Even though this method has been shown to be effective in defining clear species groups, it has not yet been used diagnostically for all the members within the genus *Burkholderia*. To date, there are eight formally named genomovars with genomovar IX (*B. pyrrocinia*) awaiting confirmation; only six of these can be detected diagnostically through *recA* PCR amplification (Mahenthiralingam *et al.*, 2000a; 2000b). Still awaiting an allocated genomovar is *B. fungorum* and it has been possible through this study to generate a *recA* sequence from this species (see figure 4.18). The generation of a 330bp fragment through the use of specially designed generic primers could only be seen in the *B. fungorum* strain tested, no other bands from mis-priming events could be seen. The sequence generated provided important genetic information to make it possible to design a set of diagnostic primers specific to *B. fungorum*, to suggest that it could be nominated as genomovar X. Primers were also designed for *B. sacchari* with a sharp and intense 248bp amplicon being visualised on agarose gel. However, this amplicon was seen in this species as well as *B. cepacia* type strain 2993, *B. ambifaria* and *B. multivorans*. Though this amplicon was appears to be specific for the *B. sacchari* strain. The generation of the 248bp product in the other species may have been due to a mis-priming event giving false positive results for *B. sacchari*. It was not possible to generate species-specific primers for *B. caledonica* due to the *recA* sequence being too short to define an unique area of heterogeneity (see figure 4.20).

It was not possible to design species-specific primers for the two representative novel *Burkholderia* isolates used in this part of the study (see figure 4.19). Only YWS-03 (*Ulmus* sp.) generated a single band from the primers used in this analysis. However, other species and isolates also produced the same fragment as well as other sized fragments with the primer set. It would be a huge assumption to say that the single band generated from YWS-03 would be unique to this isolate. It would be more prudent to design another primer set based on a longer sequence of *recA* gene from this isolate containing more nucleotide mismatches for higher specificity.

In summary, it was difficult to generate *recA* sequence data from other *Burkholderia* species and novel isolates due to the regions of conserved sequence from the aligned data being minimal. The aligned *recA* data also demonstrated that the *recA* sequences between species and strains were variable. Therefore, the introduction of

novel species into any alignment could compound the difficulties in designing primers for some species of *Burkholderia*. In the case of the novel *Burkholderia* sequences it appeared that the primer sites were similar to other sequences within the genome, thus producing the multiple PCR products.

The ability to fix nitrogen is a characteristic found in some *Burkholderia* isolates, a property confirmed through the presence of *nif* genes (Estrada-De-Los Santos *et al.*, 2001; Minerdi *et al.*, 2001; Moulin *et al.*, 2001). The *nif* gene group can be found along the genome within such *Burkholderia* isolates; each *nif* gene is responsible for a part of the nitrogen fixation process. Some of the novel isolates were closely related to the nitrogen fixing strains on the 16S rDNA phylogram (see figure 4.15). Using the 16S rDNA data as an indicator it was assumed that some of the novel isolates may possess this ability as well. In previous studies the presence of the *nif*K gene was used as a marker for the nitrogen fixation characteristic; which can be found in *B. vietnamiensis*, a rice rhizospheric strain (Gillis *et al.*, 1995; Estrada-De-Los Santos *et al.*, 2001; Minerdi *et al.*, 2001). An amplicon was seen in the novel isolate CWP-01 (*Laurus* sp.) and the *B. cepacia* type strain 2993, but these proved not to be homologous to the *nif*K fragments generated from *B. vietnamiensis*.

4.22.5 DNA-DNA binding

The DNA-DNA binding assay was the final confirmation of the relatedness of the novel isolates to the various *Burkholderia* type strains used (see table 4.1). It was clearly seen that the novel isolates had higher levels of relatedness to one another than they did to the authenticated species used in this study. However, the kinetics of binding did vary when the probe DNA was switched with the bound DNA (*i.e.* probe –2993 and bound OY715 compared with bound -2993 and probe -OY715). This difference in binding values could be due to the higher availability of DNA on the bound surface than there was with the probe DNA. The probe was labelled with photobiotin and sonicated before being used in the assay. This may indicate that during the binding and sonication process, some fragments of DNA underwent binding, but these fragments may have not been labelled with photobiotin. The level of relativity or species determination is $\geq$70% (Vandamme *et al.*, 1996), but none of the novel isolates reached this level as to be characterised as one of the type strains. The binding of the novel isolates proved to be low amongst the type strains, but slightly higher amongst the other novel isolates. However, the differences between the novel

isolates were great, this suggested low level of relatedness between them. To suggest that the novel isolates should be nominated as a new species would be a large assumption considering DNA-DNA binding with other representative species within the *Burkholderia* genus have not been made. Before nomination of a novel species, representative species of the *Burkholderia* genus should be assayed using this method to ensure that the candidate isolates are truly novel.

Further study is required to enable speciation of the novel isolates described during this study. Should they show low binding values for all of the *Burkholderia* species, then a novel species nomination could be proposed. DNA-DNA binding is a valuable tool in the taxonomy of *Burkholderia* and other micro-organisms. Despite this method being laborious, (due the large amount of good quality DNA needed) it was the final tool used to determine the relatedness of novel isolates to known *Burkholderia* species. However, it should be noted that analysis of each isolate should be repeated to ensure reproducibility and statistically correct values.

4.22.6 Summary

Due to its genomic plasticity, despite the use of a variety of methods categorising *Burkholderia* isolates into precise groups was difficult; this finding has been confirmed by other researchers (Lessie *et al.*, 1996; Coenye *et al.*, 2001c; Coenye *et al.*, 2001d; Fiore *et al.*, 2001). The high variability compounded with the large size of the *Burkholderia* genome has caused difficulties in taxonomic grouping (Lessie *et al.*, 1996). Even conserved genes such as *recA* and the 16S rDNA gene have shown heterogeneity within the genus. However, the *recA* gene has proved itself to be a useful tool in categorising some of the species into genomovars, it is clear that some members of the genus are yet to be characterised to the species level. The combination of molecular methods has helped to group isolates together into clusters. Through the use of various molecular methods some conclusions can be drawn on the relatedness of the novel isolates and established species within the genus *Burkholderia*.

The woodland rhizospheric isolates did not reveal any form of plant or habitat specificity from the whole genome DNA analysis (BOX, ERIC and AFLP). The 16S rDNA gene analysis confirmed that a group of woodland *Burkholderia* isolates could

be defined. However, species-specific characteristics and pathogenicity were hard to define through this method of analysis.

The novel *Burkholderia* isolates CWP-01 (*Laurus* sp.) and YWS-03 (*Ulmus* sp.) examined under *recA* gene sequence analysis proved to be similar to the *B. cepacia* genomovar IIIa isolates. However, further analysis was required to clarify whether these are truly *B. cepacia* genomovar IIIa or if they were a part of the newly characterised genomovar IIIc and IIId groups.

The DNA–DNA binding assay revealed that the novel isolates were different from established species within the *Burkholderia* genus. This assay also revealed that genome heterogeneity could also be seen between the novel woodland isolates.

CHAPTER 5

BIOREMEDIATION OF POLYAROMATIC HYDROCARBONS

5.0 Background

Polyaromatic hydrocarbons (PAHs) are chemicals that can be found throughout the environment. It was hoped that some environmental isolates of *Burkholderia* would exhibit bioremedial properties as a result of environmental exposure to PAHs.

5.1 Bioremediation of polyaromatic hydrocarbons by novel *Burkholderia* isolates

The novel *Burkholderia* woodland isolates were grown on minimal mineral medium supplemented with 0.5% glucose for a period of 2 days then starved in mineral medium without any glucose or other carbon sources. Through the starvation of the isolates it was hoped that the bacteria would utilise the PAHs as a carbon source to grow and survive on the medium. The isolates were left for a total of 14d at 28°C in the dark and growth was periodically checked. The growth of the bacteria was compared with positive and negative controls. The presence of growth on the supplemented plates indicated tolerance of the PAH by the isolate or that the PAH was being utilised by the isolate.

The PAHs used in this investigation were pyrene, phenanthrene and PAH mix number 9 (PAH 9) (see chapter 2 section 2.30 for contents of PAH mix no.9). The isolates showing good growth on these supplemented plates were then subsequently used in a liquid enrichment study.

During this study, the levels of growth were monitored over a period of 14 days after which the PAH was extracted from the liquid phase and examined under GCMS (Dr Alwyn Fernandes and his team at CSL carried out the GCMS analysis).

The following isolates were used in the liquid enrichment study: C3AIM *B. graminis* (*Graminacae* sp.), C4DIM *B. graminis* (*Graminacae* sp.), C5AIM *B. graminis* (*Graminacae* sp.), CWS-03 *Burkholderia* sp. (*Platanus sp.)*, GR34 *Burkholderia* sp., KPS-02 *Burkholderia* sp (*Quercus* sp.), KPS-05 *Burkholderia* sp. (*Fagus* sp.), YWS-04 *Burkholderia* sp. (*Ulmus* sp.), YWS-07 *Burkholderia* sp. (*Betula pendula*) and YWS-08 *Burkholderia* sp. These isolates were chosen from a previous solid media selection experiment. The isolates which showed signs of growth on a PAH supplemented agar plate were chosen for the liquid enrichment experiment.

Unfortunately, due to experimental error, the levels of PAH were found to be too low for GCMS analysis. The levels were so low that it was difficult to detect between baseline PAH levels found naturally within the environment from the PAH added. The extraction process and the presence of the glucose resulted in low amounts of PAH being extracted. It was observed that the medium which contained glucose did not form an emulsion when the extraction solvent was used. Instead it formed a gelatinous layer which hindered the extraction of the solvent layer containing the PAH.

Table 5.1 Growth of *Burkholderia* isolates on PAH enriched minimal mineral agar

The levels of bacterial growth were determined visually by comparing the PAH enriched plate to minimum mineral plate without a carbon source (negative growth control) and supplemented with 1% glucose (positive growth control).

	PAH9 1µg	PAH 9 500ng	PYR 500ng	PYR 250 ng	PHEN 500ng	PHEN 250ng	PAH9 1µg + GLU	PAH9 500ng + GLU	PYR 500ng + GLU	PYR 250ng + GLU	PHEN 500ng + GLU	PHEN 250ng+ GLU
C3AIM	+	++	++	++	+	+	++	++	+++	+++	++	+++
C4DIM	+	++	++	++	+	+	+++	+++	+++	+++	++	+++
C5AIM	+	++	++	++	+	++	+++	+++	++	++	++	+++
CUP	+	+	-	+	+	+	-	+	+	++	++	+
CWL-01	-	+	-	-	-	+	++	++	++	++	+	+
CWP-01	-	-	-	-	-	-	-	-	+	++	-	-
CWP-02	+	+	+w	+	+	+	+	+	+	++	+	++
CWR-02	-	-	-	+	-	-	-	+	+	+	-	-
CWS-02	+	++	+	+	++	++	+	++	+	++	+	+++
CWS-03	+	+	-	++	+	+	+	++	++	+++	++	+++
GR34	+	+	-	+	+	++	++	++	++	+++	++	++
HWL-10	+	+	+	+	+	+	++	++	+++	++	++	++
HWL-11	+	+	-	-	+	+	++	++	++	++	+	++
HWP-12	-	++	+	+	+	+	++	++	++	++	++	++
HWP-13	+	+	+	+	+	-	+	+	+	+	+	+
HWP-15	-	+	-	+	+	+	++	+	++	++	++	++
HWP-16	+	++	++	++	++	++	++	++	+	+++	++	+++
HWS-12	-	-	-	-	-	-	-	-	+	+	+	-
HWS-20	+	+	+	+	+	+	+	++	++	++	+	+
HWS-21	-	-	-	-	-	+	++	++	++	+++	++	+
HWS-22	+	+	-	+	+	+	+	+	++	++	+	++
HWS-24	-	+	-	-	-	-	++	++	++	++	+	+
HWS-26	-	+	-	-	-	-	++	+	++	++	+	+
HWS-28	-	-	+	-	+	+	+	++	++	++	+	++
HWS-29	-	+	+	+	+	+	++	++	++	++	+	++
HWS-30	+	+	+	-	+	+	+	+	++	++	+	+
HWS-31	-	-	-	+	+	+	+	++	++	++	+	++
HWS-33	++	++	+	++	++	++	+	++	++	++	+	+

	PAH9 1µg	PAH 9 500ng	PYR 500ng	PYR 250ng	PHEN 500ng	PHEN 250ng	PAH9 1µg + GLU	PAH9 500ng + GLU	PYR 500ng + GLU	PYR 250ng + GLU	PHEN 500ng + GLU	PHEN 250ng + GLU
HWS-36	-	+	-	+	+	+	+	+	++	++	++	++
HWS-37	++	++	+	+	+	+	++	++	++	++	+	++
HWS-39	+	+	+	+	++	+	+	+	+	+	++	++
HWS-41	-	-	-	-	-	+	-	+w	+	+	+	+
HWS-43	++	+	++	++	++	+++	-	+	+	+++	++	+++
HWS-44	++	++	+	++	++	++	++	++	++	++	++	++
KPS-02	+	+	+	++	+	++	++	++	+	+++	+++	+++
KPS-05	+	++	+	++	+	++	+	++	++	+++	++	++
KPS-07	+	+	+	+	++	++	++	+	++	+++	++	++
J2315	+	+	+	+	+	++	+	+	+	++	+	+
OY715	-	+	+	+	-	+	++	++	++	++	++	++
RW36	+	+	-	-	-	-	+++	+	+	++	++	++
S512	+	+	-	-	-	-	++	+	++	++	++	++
YWS-01	+	+	-	+	+	+	++	++	++	+++	++	++
YWS-02	-	+	+	+	+	+	+	+	++	++	++	+
YWS-04	++	++	++	+++	++	++	+++	++++	+++	++++	+++	+++
YWS-05	-	-	-	+	-	-	+	+	+	++	+	+
YWS-07	++	++	++	+++	++	++	+++	++++	++	+++	+++	+++
YWS-08	+	+	-	+	+	+	+++	+++	+++	+++	++	++
YWS-11	-	-	-	-	-	-	-	-	-	+	-	-
YWS-12	+	+	-	+	++	++	++	+	++	++	+	++
YWS-15	+	+	-	+	+	+	++	++	++	++	+	++
YWS-22	-	-	-	-	-	-	++	++	++	++	++	++
2993	+	+	+	+	+	++	+	++	+	+	+++	+++

Scale of growth

+w weak growth + light growth ++ good growth +++ very good

++++ same as positive control

5.2 DISCUSSION OF BIOREMEDIATION STUDY

PAHs can be found within most soils and can even be detected within the air (Dr Alwyn Fernandes- a personal communication) due to the combustion of natural fuels such as coal; therefore exposure to these chemicals is not a rare event (Wang *et al.*, 2003). However, due to their instability in the presence of UV, the persistence of these chemicals within the top layer of soil and surfaces of plants could be short lived. The presence of the PAHs within the environment indicated that some environmental isolates could have the metabolic ability to utilise these chemicals. This suggested that if the ability to metabolise PAHs is present within an isolate then the appropriate genes for the bioremediation of these chemicals would be present.

The ability to grow on the solid medium supplemented with PAH was investigated further through the use of liquid enrichment cultures. This enabled the extraction of the PAH from the liquid medium for further analysis. Through GCMS it was hoped that it would be possible to determine the metabolites formed by the *Burkholderia* isolates through the breakdown of the PAHs. This would also assist in determining whether the breakdown of the PAHs resulted in the safe biodegradation without the production of a more persistent and toxic by-product (Timmis & Pieper, 1999).

The artificial environment created by supplying an alternative carbon source in the mineral medium would 'encourage' the bacteria to utilise the PAHs. The presence of bacterial growth and survival in the agar enrichment could result from an alternative hypothesis. The bacterial cells could have survived by utilising the nutrients from 'non-viable lysed' bacterial cells. It was assumed that due to the competition for nutrients some bacterial cells might have not survived on this minimal nutrient medium. Through bacterial cell death and eventual lysis of the cell, the nutrients from the bacterium were then released into the media for the remaining viable bacteria to utilise. Alternatively, the isolates were surviving the minimal medium by utilising the PAHs as a sole carbon source or co-metabolising PAHs with the glucose present within the agar.

Unfortunately, due to the failure of the liquid enrichment experiment the assumption of the PAH being utilised by the bacteria could not be confirmed or rejected. However, the

assumption that some of the novel isolates could at least survive in media containing PAHs is supported by the presence of bacterial growth.

However, through the liquid enrichment analysis, the extracted PAH levels were too low to be analysed due to some experimental error and the volatile nature of the chemicals used. Therefore, it was not possible to determine the by- products from the metabolism of PAHs, pyrene and phenanthrene. This experiment was not repeated because of time constraints and difficulties in obtaining further GCMS analysis prevented further modification of the experiments and testing of novel isolates for bioremediative properties.

CHAPTER 6

GENERAL DISCUSSION

6.0 Background

The main task of this study was to devise a taxonomic method capable of categorising novel *Burkholderia* and known species of *Burkholderia* into more meaningful groups. This chapter will assess the methods used to classify *Burkholderia* according to habitat or plant specificity.

6.1 Taxonomy of the genus *Burkholderia*

From this study it was clear that the single classification techniques have a limited ability to discriminate between species within the genus *Burkholderia*. It has been difficult to examine the taxonomic diversity of *Burkholderia* within woodland habitats due to the inherent difficulty in defining key characteristics between them.

For example, fatty acid profiling had shown itself to be useful to the genus level but to the species level it was not reliable, especially with the recent additions of new *Burkholderia* species *B. ambifaria*, *B. caledonica*, *B. fungorum* and *B. sacchari*. The FAPs of these species were not contained within the library, therefore it was difficult to use their FAPs as a method of species and cluster identification with the novel woodland isolates. However, fatty acid profiling did provide an interesting and robust method for characterisation of the novel woodland isolates as *Burkholderia* and to investigate the differences in phenotypic characteristics. The data generated from the novel woodland isolates could be compared against those from the *Burkholderia* reference strains.

The API carbohydrate assay could only define the nutritional abilities of each isolate and was insufficient to categorise the isolates into any meaningful or common groupings. The carbohydrate profiles varied between the type of novel isolates tested; none of the woodland isolates had any common carbohydrate results. However, the *Quercus* (oak)

isolates had similar carbohydrate profiles on the API 20NE with caprate, citrate and cytochrome oxidase tests varying between them. The *Fagus* (beech) isolates also processed similar results with variation in the p-nitrophenyl-β-D-galactopyranoside, glucose, citrate and cytochrome oxidase tests.

Novel isolates CWP-03, HWP-20, HWS-03 and HWS-44 all had similar carbohydrate profiles to the *Allium cepa* (onion) isolates OY715. However, when cross-referencing these isolates on the dendrograms produced by the BOX, ERIC and AFLP PCR fingerprints, these isolates were not found to be in close proximity. This finding was also confirmed when pin pointing the position of these isolates on the 16S rDNA phylogram. This indicated that carbohydrate profiles from the novel woodland *Burkholderia* isolates could not be used to group them according to species of plant or plant pathogenicity.

The use of random repetitive sequences within the genome (AFLP, BOX element and ERIC) and the combination of these methods generated some species-specific groupings within the *Burkholderia* genus. However, they failed to define human pathogenic strains or habitat-specific clusters during this study. The woodland isolates when combined with the data generated by Jane Richardson appeared to show some woodland habitat specificity. However, plant specificity could not be seen within the clusters. Until all the DNA fingerprint data from the two studies are generated by the same operator at a single time point, the woodland clusters are only a tentative taxonomic grouping.

A discriminative taxonomic grouping was achieved through the sequencing of the conserved 16S rDNA and *recA* genes, where key characteristic groups have been defined. The novel isolates appeared to differ and formed clusters separate from the *B. cepacia* complex isolates in these assays, this showed that 16s rDNA gene PCR had the power to discriminate between the two groups. The formation of characteristic clusters opened up other avenues of investigation, for example, the *nifK* gene assay was used to determine whether any of the novel isolates clustered closely with known nitrogen fixers possessed the potential for nitrogen fixation. The novel isolate CWP-01 was chosen as it was isolated from *Laurus* sp. root; it also clustered closely with woodland root and rotting wood isolates RW36, WR19X, RW6 and S516 on the 16S rDNA phylogram. Through these informal criteria it was hoped that CWP-01 could be shown to have nitrogen fixing properties and so a search for the *nifK* gene was carried out on this novel isolate and

known nitrogen fixing species *B. vietnamienesis* and a *Klebsiella* mutant containing a *nifK* gene construct. Testing for the presence of the *nifK* gene within the novel *Burkholderia* isolates would indicate whether this property was true to this particular cluster. Novel isolate CWP-01 was not found to possess the *nifK* gene, thus suggesting that the other novel isolates were unlikely to fix nitrogen. However, this assumption should be confirmed through further *nif* gene analysis as the novel isolates may not be in possession of the complete *nif* gene operon and may only possess some of the *nif* genes.

The potential bioremedial isolates were chosen by their closeness to the known bioremedial strains such as LB400, N3P2, N2P5, N2P6, CRE7 and Dha-5 amongst others. Some of the novel isolates that were distantly clustered were also included to compare them against any bioremedial activity displayed by the potential bioremediators. The bioremediation studies failed to reveal bioremedial properties for PAHs from the novel isolates contained within the 16S rDNA group of bioremediators. This was due to the liquid enrichment experiments being unsuccessful. It is yet to be confirmed whether the 16S rRNA gene sequencing was a definitive method to indicate specific properties such as bioremediation or nitrogen fixation. Through such comparative studies it could be possible demonstrate that the novel *Burkholderia* isolates which did not grow on PAH enriched media, may only have had a saprophytic existence through utilising nutrients from lysed bacterial cells (Sermswan *et al.*, 2001).

In previous studies, the prevalence of the novel *Burkholderia* isolates appeared to be higher in rhizospheric samples than in bulk soil samples (Miller *et al.*, 2002; Estrada-De Los Santos *et al.*, 2001; Fiore *et al.*, 2001). This finding was also supported in several studies where some species within the *Graminacae* family appeared to support high populations of *Burkholderia* isolates (Dalmastri *et al.*, 1999). During this study the numbers of *Burkholderia* were higher in rhizospheric soil samples then in non-rhizospheric or bulk soil samples such as the samples from Langlands (see chapter 3). *Burkholderia* isolates have been found both externally on root surfaces and internally within root tissues (Gillis *et al.*, 1995; Dalmastri *et al.*, 1999; Coenye *et al.*, 2001c). Some of the endophytic strains of *Burkholderia* have also been shown to possess nitrogen fixation abilities, which promoted plant growth (Coenye *et al.*, 2001d; Richardson *et al.*, 2002). The external colonisation of roots has been shown to improve plant survival through plant protection properties such as the generation of antibiotics and

siderophores. Although these properties can been seen as beneficial and could encourage the use of certain *Burkholderia* strains; the use of such bacteria, often referred to as plant promoting bacteria, could also be seen as a threat to human health should human pathogenicity also be a characteristic. For these reasons, no *Burkholderia* strains have been fully approved for use as biocontrol agents. Before approval or registration, such *Burkholderia* strains will have to undergo rigorous risk analysis to show that there are no risks to human or animal health or to the environment. Plant pathogenicity of these biocontrol strains to crops such as onions will also have to be assessed. For human health there are two main concerns, long-term survival of a pathogenic form within the environment and risks to operators preparing or administering the product to crops or land. Since exposure to airborne bacteria during these processes will be high it would be prudent to investigate the risks to human health during exposure. This study examined one of these human virulence factors, the *cblA* gene associated with the attachment of *Burkholderia* to the epithelial lining of the respiratory system.

The final molecular method for defining novel isolates is the DNA-DNA binding assay where the whole genome was compared to reference strains through the measurement of re-binding values. This has shown to be useful in determining relativity of the different DNAs and has revealed the genetic diversity which can be found within the genus *Burkholderia*. This genetic diversity could account for the difficulty of finding one single method to categorise all of the species within this genus, or to cluster the novel isolates according to habitat or plant specificity. However, this has indicated that although classification by the comparison of the whole genome is difficult, classification through comparing gene data such as *recA* and 16S rDNA could be more fruitful.

The number of species in the genus *Burkholderia* is continuing to expand as more isolates are being isolated from clinical samples and from the environment. The methods used to further separate the novel species from the older established species are now used in combination to generate a higher level of discrimination.

6.2 Taxonomy of novel *Burkholderia* isolates

The environmental sampling carried out during this study would have selected *Burkholderia* isolates which were least likely to be human pathogenic strains. Therefore, it was expected that a woodland cluster might be revealed in some of the dendrograms or phylograms created during this study. However, within the woodland isolates there was little evidence of habitat or plant species specificity. Because of the failure of species determination of the novel *Burkholderia* isolates through the use of biochemical tests and various molecular assays, a polyphasic approach to taxonomy is required to categorise and identify such a genetically diverse genus of bacteria. Over the years researchers have used several combinations of molecular methods to try and form a comprehensive categorisation of this genus (Vandamme *et al.*, 1996, Coeyne *et al.*, 1999b, Vandamme *et al.*, 2002a).

During the course of this study it has become apparent that the molecular methods currently used are inadequate in generating species-specific or character specific groups within the genus *Burkholderia*. The *Burkholderia* genome is known for its diversity and 'plasticity', which makes the task of taxonomic grouping difficult, especially when using whole genome classification techniques such as BOX, ERIC PCR and DNA-DNA binding.

The genomes of some *Burkholderia* species such as *B. cepacia*, *B. gladioli*, *B. hospita* and *B. terricola* have been reported to vary in size. They also been shown to have the ability to obtain genetic material from other micro-organisms within the clinical or soil environment (Wigley & Burton, 2000; Goris *et al.*, 2002). It has been reported in previous studies that *Burkholderia* species are nutritionally and ecologically diverse (Richard *et al.*, 1981; Gillis *et al.*, 1995). These abilities have resulted in the heterogeneous genetic profiles generated from the environmental isolates and consequently constitute a difficult taxonomic grouping. The ability to obtain extra genetic material from the environment could be possible with the novel woodland isolates which could explain the diversity of the isolates from the same species plant samples.

During this study both phenotypic and genotypic aspects of novel woodland isolates have been investigated. The relationship of these novel isolates to established species has been closely scrutinised. The habitat specificity of the novel *Burkholderia* isolates has also

been investigated to determine whether there was any environmental influence on the genetic profile. Despite using several methods of phenotypic and genotypic profiling, it was not possible to define clear habitat-specific clusters. Some species-specific clusters were starting to emerge, but until more novel woodland isolate and *Burkholderia* species are subjected to taxonomic profiling it would only be an assumption. Should more woodland isolates be tested and characterised, a species-specific cluster containing only woodland *Quercus* sp. (oak) isolates could possibly be formed. Including more *Quercus* sp. (oak) isolates for comparison could result in the species *B. multivorans* and *B. stabilis* 'falling out' of the woodland cluster and into a cluster more characteristic of these two species.

The clinical isolates tested during the course of this study were found within clusters which also contained woodland isolates. None of the taxonomic groupings formed through the various methods used revealed an exclusive cluster containing only clinical *Burkholderia* isolates. Should *Burkholderia* species be released into the environment for biocontrol or bioremedial use, the quick and accurate detection of human pathogenic strains would be important to CF patients, as mortality rates are high. To calculate or predict the likelihood of a non-pathogenic *Burkholderia* becoming a pathogenic *Burkholderia* a more robust taxonomic grouping is required. This could be achieved through the combination of methods to create a more meaningful grouping system. However, none of the methods used during this study were able to separate the environmental isolates from the clinical isolates. The need to discriminate between plant pathogenic strains is also required should the biocontrol or bioremediative strains be released into non-contained areas. The risks posed by this wide spread use of such strains to cash crops like onions will also need to be assessed.

The constant evaluation of current taxonomic methods is required to improve the detection, categorisation and epidemiology of all *Burkholderia* as it is showing itself to be a useful but potentially dangerous micro-organism.

Previously, use of the 16S rDNA gene sequence was successful in generating some taxonomic grouping of the clinical, environmental strains and novel isolates (LiPuma *et al.*, 1999). During this study it was evident that the novel woodland isolates clustered together, but to what extent they related to established *Burkholderia* species remained

unclear. The level of discrimination of the 16S rDNA gene sequence was limited and the differentiation of species or genomovars has not been possible with this method. The properties of specific groups highlighted within the 16S rDNA gene phylogram such as nitrogen fixation, bioremediation were also inconclusive for the novel woodland isolates.

The *recA* gene is one of the genes responsible for the repair and the recombination of DNA. The *recA* gene sequencing is a robust method that has been successful in the differentiation of most of the *Burkholderia* species into closely related genomic species or genomovars. The *B. cepacia* complex at present contains nine genetic species or genomovars. The *recA* gene is becoming a commonly used classification technique in determining key groups within the *B. cepacia* complex. At present there are 8 diagnostic *recA* primers available for the grouping of *Burkholderia* species into genomovars. However, it has been shown that this method can sometimes generate cross-reactions and false positive results for genomovar I, IIIa, IIIb and *B. pyrrocinia* isolates tested (Vermis *et al.*, 2002). It was suggested that the genomovar I *recA* assay should be improved in regard to the environmental isolates (Vermis *et al.*, 2002). This was because the low sensitivity of the *recA* PCR may have had difficulty in distinguishing between the environmental isolates. Increasing the sensitivity for genomovar I could result in the formation of subgroups as in genomovar III. Another reason for the further improvement to the *recA* gene sequencing method is that genomovar IIIa has been reported to contain only clinical isolates (Mahenthiralingam *et al.*, 2000a, 2000b; Coenye & LiPuma 2002; Vermis *et al.*, 2002; Chung *et al.*, 2003). Through this study, the *recA* analysis has revealed that two of the novel woodland isolates could possibly be contained within this group. This may also indicate that a further division may be possible within this particular genomovar to accommodate the environmental isolates. When grown at 28°C and 37°C CWP-01 displayed weak growth at the higher temperature. YWS-03 was not tested for growth at 37°C as only a selection of novel isolates were chosen for this study. However, when referring to the 16S rDNA phylogram closely clustered isolates YWS-05 and YWS-07 did not display growth at 37°C. This could indicate that YWS-03 would also display weak growth at 37°C as well. It should be noted that the two novel isolates contained within this cluster can not be ruled out as human pathogenic strains due to their lack of growth at 37°C as environmental isolates have been shown to cause foot rot (Taplin *et al.*, 1971). Further tests may reveal that these novel isolates may belong to the newly proposed genomovars IIIc and IIId.

6.3 Origins of novel environmental isolates

The monitoring for the presence of virulence genes such as the *cblA* gene and the *Burkholderia cepacia* epidemic strain marker (BCESM) have been used in the initial determination of human pathogenicity (Mahenthiralingam *et al.*, 1997; Richardson *et al.*, 2001; Sajjan *et al.*, 2002; LiPuma *et al.*, 2002), though it has been reported that the *cblA* gene has been found in one environmental isolate of *Burkholderia* (Richardson *et al.*, 2001). However, as these genetic markers have not been found in all human pathogenic strains, other avenues must be investigated to provide a more reliable and conclusive method for indicating human pathogenicity. In this study, none of the novel isolates tested contained the *cblA* gene, however, this does not exclude them as non-pathogenic strains. Other virulence factors such as the BCESM, pathogenicity islands, type III secretion systems should also be tested for. It would also be useful to gain some *recA* gene data from the novel isolates to see if they clustered closely with human pathogenic genomovars.

Bacterial evolution is an important issue in the taxonomy of *Burkholderia*, the capacity of a strain to become a threat to human and plant health needs to be assessed. Alongside this, the natural selection of beneficial strains from contaminated land needs to be investigated. Through environmental pressure it has been shown that genetic changes improve survival rates of the organism (Friedrich *et al.*, 2000). Delineation of the evolutionary lineage and background could help avoid large-scale use of a potentially dangerous strain of *Burkholderia* in the environment.

During this study it was clear that existing taxonomic methods were insufficient in determining the potential harm a woodland isolate could cause should it be released into the environment for biocontrol or bioremedial uses. The consequences of widespread application and use of a high concentration of *Burkholderia* within the environment needs to be assessed. The ability of any modified strains to obtain extra genetic material from other soil bacteria (such as antibiotic resistance genes) or from human pathogens will also need to be investigated. Consequences from the release of *Burkholderia* into the environment, which could affect the natural rhizospheric and soil ecology, must also be considered.

Methods that focus on relatively short regions of the bacterial genome such as the *recA* and 16S rDNA genes seem to be more successful when trying to establish taxonomic groupings within this complex genus. Using a combination of genes could be the key to resolving the problems with *Burkholderia* taxonomy. The use of a group of 'house keeping' genes in creating a comprehensive taxonomic grouping of the *Burkholderia* genus and the novel isolates found during the course of this study will need to be investigated further.

In conclusion, the potential benefits of *Burkholderia* are vast, but until the classification of this genus is more reliable and definitive, its commercial use should be limited to small controlled areas.

CHAPTER 7

FUTURE WORK

Isolation of novel isolates from the environment

The isolation of novel isolates from specific habitats is required in order to consolidate the data already generated from this study. The habitat and species specificity was starting to emerge from the phylograms and dendrograms generated throughout this study. However, the numbers of strains and isolates were too low to confirm any kind of plant species specificity. Further environmental testing will confirm or reject any of the emerging species- specific groupings found during this study.

16S rDNA gene sequencing

Continued analysis of novel environmental isolates will be useful in determining possible characteristics and cluster formation. Though the taxonomic level of discrimination based on the 16S rDNA sequence was limited, it has proven itself to be an important method in the polyphasic taxonomy of the *Burkholderia* genus.

RecA gene sequencing

The *recA* gene sequencing has proven itself to be a useful tool in the allocation of *Burkholderia* isolates into genomovars. The genomovar allocation through *recA* gene sequence analysis is required for the novel woodland rhizospheric *Burkholderia* isolates. Through this analysis it may be possible to determine a tentative genomovar allocation or to indicate the emergence of a novel species. At present, there are only 9 genomovars within the *B. cepacia* complex that can be clearly defined through the PCR analysis of this gene and subsequent sequence analysis.

Improving genomovar allocations and characteristic specificity

Taxonomy through the use of highly conserved genes such as the 16S rRNA and *recA* genes have proven to be discriminative. However, the power of discrimination is limited and the diversity of the *Burkholderia* genome requires a method or a combination of methods which is more powerful. By combining the sequence data of several different 'house-keeping' genes it may be possible to generate a higher and more discriminative form of taxonomic grouping. AFLP has been shown to generate an extremely high level of discrimination within the species of *Burkholderia*. However, this level of discrimination was so high that most isolates were contained within separate clusters. The AFLP analysis needs to be modified to produce an optimum level of discrimination to generate a working taxonomic grouping.

DNA-DNA binding

In a further study a representative type strain of each genomovar of *Burkholderia* should be assayed against the novel woodland isolates. This might determine whether the novel woodland isolates should be given a novel species allocation.

Bioremediation of complex chemicals by *Burkholderia* isolates

The study of the potential for bioremediation of PAHs in liquid culture was unsuccessful. An investigation of the breakdown of various complex chemicals and the mechanisms used would be useful in determining the benefits of *Burkholderia* as a bioremediator. For example, the ability of *Burkholderia* to break down pesticides into harmless chemical by-products would help minimise contamination events in rivers. This could also have other benefits such as reducing the amount of pesticide residues on vegetables ingested by consumers. In conjunction with the polyphasic taxonomic studies it would also assist in monitoring the potential human health risks of *Burkholderia* as bioremediator.

APPENDICES

A1 MEDIA

Pseudomonas cepacia azelaic tryptamine (PCAT) agar / 1litre (Burbage & Sasser, 1982)

Azelaic acid (Sigma- Aldrich)	2.0g
Tryptamine (Sigma- Aldrich)	0.2g
$MgSO_4$(BDH)	0.1g
K_2HPO_4 (BDH)	4.0g
KH_2PO_4 (BDH)	4.0g
Yeast extract (Oxoid)	0.02g
Agar (Oxoid)	15g

Dissolve $MgSO_4$ in distilled water and heat solution. Add azelaic acid and stir until dissolved and add remaining ingredients then autoclave at 121°C for 20min.

Nutrient agar / 1litre

'Lab Lemco' powder (Oxoid)	1.0g
Yeast Extract (Oxoid)	2.0g
Peptone (Oxoid)	5.0g
NaCl (Rose Chemicals)	5.0g
Agar (Oxoid)	15.0g

Adjust to pH 7.4 and sterilise by autoclaving at 121°C for 20min.

Trypticase soya broth agar (TSBA) / 1litre

Trypticase Soya Broth (Becton Dickinson)	30g
Agar (Oxoid)	15g
Demineralised water	1l

Adjust to ph7.4 and sterilise by autoclaving at 121°C for 20min.

TAE agarose gel (1%)

Agarose (Boehringer Mannheim)	0.8g
1x TAE Buffer	80ml

Boil at 100°C for 5min until agarose is melted. Cool to 65°C before casting onto a gel case.

GeneScan gel

SDW	18.2ml
10x TBE	3.5ml
Urea (BDH)	12.6g
Long ranger (Acrylamide)	3.5ml
Fresh 10% Ammonium sulphate (APS)	175µl
TEMED (Amresco)	24.5µl

After casting gel, leave for 45min at RT°C to allow the gel to fully polymerise before use.

Luria-Bertani (LB) broth / 1litre

Tryptone (Oxoid)	10g
Yeast Extract (Oxoid)	5g
NaCl (Rose Chemicals)	5g
Distilled water	1l

Adjust to pH7.4 and sterilise by autoclaving at 121°C for 20min.

Luria-Bertani (LB) agar/ 1litre

Tryptone (Oxoid)	10g
Yeast extract (Oxoid)	5g
NaCl (Rose Chemicals)	5g
Agar (Oxoid)	15g
Demineralised water	1l

Adjust to pH7.4 and sterilise by autoclaving at 121°C for 20min.

Ampicillin Stock Solution / 1ml

Ampicillin (Sigma-Aldrich)	50mg
Demineralised water	1ml

Filter sterilise antibiotic solution and store at -20°C

Ampicillin Supplemented LB Agar

To 500ml of LB agar at 50°C add:

Ampicillin (50mg/ml) (Sigma-Aldrich)	1ml
IPTG (1M) (Biogene)	50µl
X-gal (50mg/ml) (Promega)	500µl

Glycerol broth for bacterial stocks

LB broth	28ml
Glycerol (BDH)	2ml

Sterilise by autoclaving at 121°C for 20min.

SOC broth

Tryptone (Oxoid)	2.0g
Yeast extract (Oxoid)	0.5g
NaCl (Rose Chemicals)	0.05g
Demineralised water	95ml

Adjust to pH7.4 and sterilise by autoclaving at 121°C for 20min.

Adjust to pH7 by adding 5N NaOH. Adjust final volume (100ml) by adding demineralised water and autoclave at 121°C for 20min. Before use add 1ml of 2M Mg^{2+} stock (filter sterilised) (1M $MgCl_2$ / 1M $MgSO_4$) and 1ml of sterile 2M glucose solution (filter sterilised).

Glucose-yeast extract-calcium carbonate-agar (GYCA)

Demineralised water	3l
D-glucose (BDH)	30g
Yeast extract (Oxoid)	15g
CaCO3 – light powder (BDH)	90g
Agar No.3 (Oxoid)	60g

Adjust to pH7.4 and sterilise by autoclaving at 121°C for 20mins.

Separation gel (12%) – sufficient for 2 gels (SDS-PAGE)

Add components in the following order

Ultra pure water	13.4ml
Separation gel buffer	10.0ml
Bis acrylamide (Sigma-Aldrich)	16.0ml
SDS (10%) (Sigma-Aldrich)	400µl
TEMED (BDH)	20µl
Ammoniumpersulphate (10%) (BDH)	140µl

Mix well and use immediately at RT°C overlay the gel with butan-1-ol to obtain a flat surface. Leave separation gel to polymerise for 1 hour, decant butan-1-ol and rinse gel border with ultra pure distilled water. Overlay gel with 3ml overlay buffer and let polymerisation continue overnight at RT°C. Decant overlay buffer and rinse gel border with ultra pure distilled water then stacking gel buffer, remove all solutions before adding stacking gel.

Stacking gel (5%) (SDS-PAGE)

Ultra pure water	10.4ml
Stacking gel buffer	5.0ml
Monomer solution (Sigma-Aldrich)	3.4ml
SDS (10%) (Sigma-Aldrich)	200µl
TEMED (BDH)	20µl
Ammoniumpersulphate (10%) (BDH)	100µl

Mix well and use immediately, insert Teflon combs and leave gel to polymerise for 1h. Once gel has polymerised rinse the wells with upper tank running buffer before loading sample for electrophoresis.

Minimal mineral medium (MM2) / 1l

To make 1l MM2 medium, add nitrogen and buffer stock solutions to a final concentration of 1X in sterile demineralised water. To this, add 500µl trace element solution.

10X Nitrogen stock

$(NH_4)_2 SO_4$ (Sigma-Aldrich)	2.37g
$CaCl_2. 2H_2O$ (Sigma-Aldrich)	0.11g
$MgSO_4. 7H_2O$ (Sigma-Aldrich)	0.246g
$NaCl_2$ (Sigma-Aldrich)	0.497g

Adjust to pH 6.8 and sterilise by autoclaving at 121°C for 20min.

10X Buffer stock / 1l

Na_2HPO_4 (Sigma-Aldrich)	1.41g
KH_2PO_4 (Sigma-Aldrich)	1.361g

Sterilise by autoclaving at 121°C for 20min.

Trace element supplement / 1l

$FeSO_4$ (Sigma-Aldrich)	2.556g
$ZnSO_4.7H_2O$ (Sigma-Aldrich)	0.100g
$MnCl_2.4H_2O$ (Sigma-Aldrich)	0.300g
$CaCl_2.6H_2O$ (Sigma-Aldrich)	0.200g
$CuCl_2.2H_2O$ (Sigma-Aldrich)	0.010g
NiCl2.6H2O (Sigma-Aldrich)	0.020g
$Na_2MoO_4.2H_20$ (Sigma-Aldrich)	5.000g
H_3BO_3 (Sigma-Aldrich)	0.300g

If required, add a few drops of 30% HCl to salts dissolve into solution. Filter sterilise trace element solution.

Minimal mineral agar / 1l

MM2	999.5ml
Agar (Oxoid)	18g

Sterilise by autoclaving at 121°C for 20min. Cool agar to 65°C before adding 500µl trace element solution.

A2 BUFFERS

50x Tris Acetate EDTA (TAE) buffer/ 1litre

Trizma base (Sigma-Aldrich)	242g
DiSodium EDTA (Amresco)	18.6g
Glacial acetic acid (Rose Chemicals)	57.1ml

Sterilise by autoclaving at 121°C for 20min.

Loading buffer for ABI Prism 377 (per sample)

	(µl)
Deionized formamide (BDH)	1.25
Blue dextran/50mM EDTA loading solution	0.25
GenScan-500 ROX size standard ladder	0.5

Tris-EDTA (TE) buffer

Tris-Cl (pH7.6) (Sigma-Aldrich)	10mM
EDTA (pH8.0) (Amresco)	1mM

5x Tris Boric EDTA buffer (TB)

Tris base (Sigma-Aldrich)	54g
Boric acid (BDH)	27.5g
EDTA (0.5M) (Amresco)	20ml

20x SSC

NaCl (Rose Chemicals)	175.3g
Sodium citrate (Rose Chemicals)	88.2g
Demineralised water	800ml

Adjust to pH7.0 with a few drops of 10M NaOH adjust volume to 1l and sterilise by autoclaving at 121 °C for 20min.

20 X SSPE

NaCl (Rose chemicals)	175.3g
NaH_2PO_4 (BDH)	27.6g
EDTA (Amresco)	7.4g
Demineralised water	800ml

Adjust to pH7.4 with 10M NaOH and adjust volume to 1l with demineralised water and sterilise by autoclaving for 15min at 121°C

Stringency washes for DNA hybridisation with P^{32}

Stringency wash 1
2x SPPE – 0.1% SDS

Stringency wash 2
1X SSPE – 0.1% SDS

Stringency wash 3
0.1X SPPE – 0.1% SDS

A3 GRAM STAINING SOLUTIONS AND STREAKING METHOD USED FOR OBTAINING A PURE CULTURE

Crystal violet solution

2%w/v crystal violet in 20% ethanol: demineralised water.

Grams iodine / 300ml

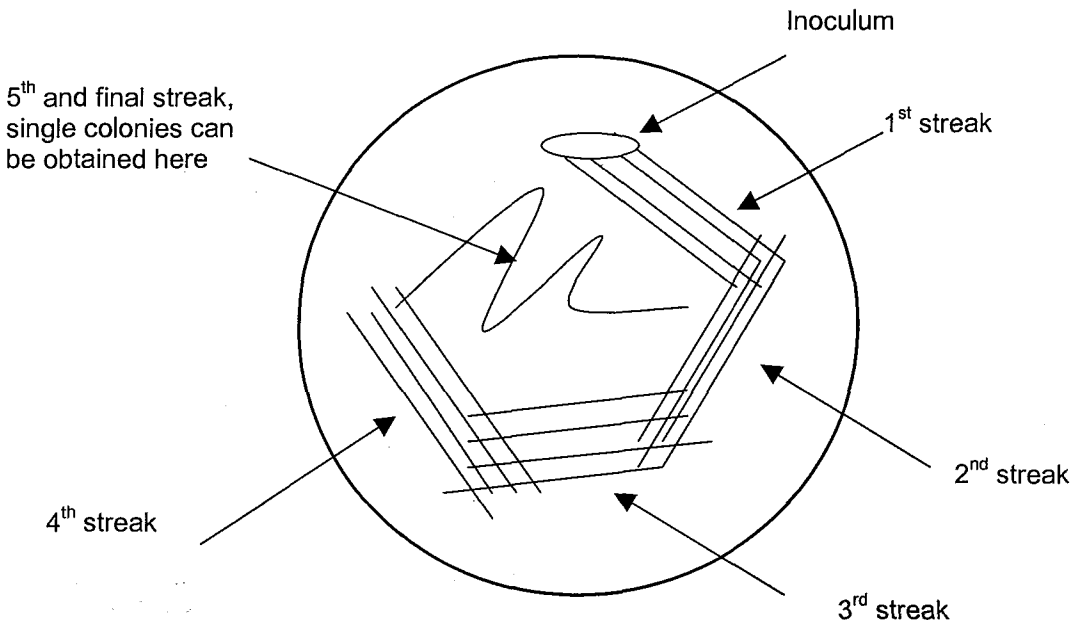
KI	2g
I_2	1g

Safranin solution

2.5% safranin in 95% v/v ethanol : demineralised water.

Streaking method used for obtain a pure culture

Using aseptic techniques a mixed culture can be separated through streaking it onto an agar plate, after each section was streaked out, the wire loop was flamed until red-hot and left to cool before being used for the next streak. This method dilutes the number of bacteria cells in each streaked section to finally obtained clean single colonies on the agar plate. The fully streaked plate is then incubated under the appropriate conditions.



A4 SDS-PAGE BUFFERS AND SOLUTIONS

Sodium phosphate buffer saline (NaPBS) / 1l

0.2M $Na_2HPO_4.12.H_2O$ (Sigma-Aldrich)	40.5ml
0.2M $NaH_2PO_4.2H_20$ (Sigma-Aldrich)	9.5ml
NaCl (Sigma-Aldrich)	8g
Demineralised water	950ml

Adjust to pH7.3 and sterilise by autoclaving at 121°C for 20min.

Sample treatment buffer (STB) pH 6.8 (SDS-PAGE)

Tris (Sigma-Aldrich)	1.5g
Mercaptoethanol (Sigma-Aldrich)	10ml
Glyercol (BDH)	20ml
Demineralised water	70ml
1M HCl (BDH)	6.8ml
4mM EDTA (BDH)	298g (4mM)

Make up to 200ml after checking pH is at 6.8. Store at 4°C.

Separation gel buffer (SDS-PAGE)

Tris (Sigma-Aldrich)	18.16g
Ultra pure water	40ml
1N HCl	37.6ml

Make up to 100ml with ultra pure water and pH Is at pH 8.78.

Stacking gel buffer

Tris (Sigma-Aldrich)	6g
Ultra pure water	25ml
1N HCl	47.3ml

Check that buffer is at pH 6.75. Adjust buffer to the final volume of 100ml with ultra pure water.

Overlay buffer (SDS-PAGE)

Separation gel buffer	25ml
Ultra pure water	75ml
SDS (10%) (Rose Chemicals)	1ml

Running buffer for lower tank (SDS-PAGE)

Tris (Sigma-Aldrich)	12g
Glycine (BDH)	57.5g
SDS (Rose Chemicals)	4g
Ultra pure water	4l

Adjust the buffer to pH 8.65

Running buffer for upper tank (SDS-PAGE)

Tris (Sigma- Aldrich)	12g
Glycine (BDH)	57.5g
Ultra pure water	4l

Store at 4°C. Just before use, add 7ml of a 10% SDS solution to 700ml of running buffer.

Tracking dye stock solution (0.05%) (SDS-PAGE)

Bromophenol blue (BDH)	50mg
Demineralised water	100ml

Dilute 1:10 in STB for SDS-PAGE.

Coomassie blue staining solution

Coomassie blue R250 (2% stock solution in distilled water) (Sigma-Aldrich)	125ml
Methanol (BDH)	500ml
Acetic acid (Rose Chemicals)	100ml
Demineralised water	375ml

Destaining solution

Methanol (BDH)	500ml
Acetic acid (Rose Chemicals)	200ml
Demineralised water	1.25l
Glyercol (BDH)	50ml

A5 OTHER SOLUTIONS

50x Denhardts solution

Ficoll (Type 400) (Pharmacia)	5g
Polyvinylpyrrolidone (Sigma-Aldrich)	5g
Bovine serum albumin (Fraction V) (Sigma-Aldrich)	5g
Demineralised water	500ml

Filter sterilise and store at -20°C.

PEG Solution

Polyethylene glycol (PEG) (Sigma-Aldrich)	2.62g
1M $MgCl_2$ (Sigma-Aldrich)	66µl
3M NaOAc (Sigma-Aldrich)	2000µl
SDW	7934µl

Sterilise PEG solution at 121°C for 15min.

A6 REAGENTS USED FOR FATTY ACID PROFILING (FAP)

Saponification Reagent (Reagent 1)

Sodium hydroxide (certified ACS)	45g
Methanol (Reagent grade)	150ml
Double distilled Water	150ml

Add water and methanol to NaOH pellets in bottle and stir until dissolved.

Methylation reagent (Reagent 2)

6.0M Hydrochloric Acid (BDH)	325ml
Methanol (Reagent grade) (BDH)	275ml

Add the acid slowly and carefully to the methanol while stirring

Extraction Solvent (Reagent 3)

Hexane (HPLC) (BDH)	200ml
Methyl tert-butyl ether (HPLC Grade) (BDH)	200ml

Add ether to hexane and stir

Base Wash Reagent (Reagent 4)

Sodium hydroxide (Certified ACS) (Sigma-Aldrich)	10.8g
Double distilled water	900ml

Add water to pellets and stir until dissolved.

Fatty acids found in *Burkholderia* species.

Shorthand Name	Systematic Name
12:0 (saturated acid)	Dodecanoic acid
16:0(saturated acid)	Hexadecanoic acid
16:1 2OH (hydroxy acid)	2- Hydroxyhexadecenoic acid
16:0 2OH (hydroxy acid)	2- Hydroxyhexadecenoic acid
16:0 3OH (hydroxy acid)	3- Hydroxyhexadecenoic acid
17: 0 cyclo (cyclopropane acids)	*Cis*-9-10-methylene hexadecanoic acid
18:0 (saturated acid)	Octadecanoic acid
19:0 Cyclo ω8c (cyclopropane acids)	*Cis*-11,12 methylene octecanoic

A7 ENVIRONMENTAL ISOLATE CODING SYSTEM FOR THE *BURKHOLDERIA* LIBRARY

C - Chun

H - Hampstead Heath, London, UK

K - Kensington Park, London, UK

Y - York, UK

W - Woodland

L - Leaf / Leaf Litter

P - Plant / Roots

R - Rotting wood

S - Soil

U - Unknown

Therefore:

CWS = Chun/ Woodland / Soil.

HWP = Hampstead Heath/ Woodland/ Plant

A8 REAGENTS FOR DNA-DNA HYBRIDISATION IN MICROTITRE PLATES.

10X Phosphate buffered saline (PBS) –500ml

Na Cl (Rose Chemicals)	40g
KCl (BDH)	1g
$Na_2HPO_4.2H_2O$ (BDH)	5.75g
KH_2PO_4 (BDH)	1g

Sterilise by autoclaving at 121°C for 20min

PBS-Mg-1litre

10X PBS	10ml
$MgCl_2.6H_2O$ (BDH)	2.03g

Sterilise by autoclaving at 121°C for 20min.

Prehybridisation solution

20X SSC	20ml
50X Denhardt solution	20ml
Denatured salmon sperm DNA solution (GibcoBRL)	2ml
SDW	58ml
Formamide (Sigma-Aldrich)	100ml

Hybridisation solution

Prehybridisation solution	100ml
Dextran sulphate (Sigma-Aldrich)	2.5g

Photobiotin Solution

Dissolve photobiotin powder (Sigma-Aldrich) to a final concentration of 500μg / ml in SDW. Aliquot 50μl into eppendorfs and stored at -4°C for up to 5 months.

Tris-HCL Saturated Butan-1-ol

Add 50ml 0.1M Tris-HCl (pH 9) to 100ml Butan-1-ol (BDH).

4-Methylumbelliferyl-β-D-galactopyranoside (4-MUG) stock solution

4-MUG (Sigma-aldrich)	10mg
N,N-dimethlyformamide (BDH)	1ml

4- Methyllumbelliferyl-β-D-galactopyranoside (4-MUG) solution for 1 plate

4-MUG stock solution	100μl
1X PBS + 1mM $MgCl_2$	10ml

Streptavidin-β-D-galactosidase solution for 1 plate

Streptavidin-β-D-galactosidase (GibcoBRL)	10μl
PBS+ 0.5% Bovine Serum Albumin (Sigma-Aldrich)	10ml

A9 API CARBOHYDRATE CODES

API 20NE Carbohydrate codes

Test	Substrate	Reaction
NO_3 / N_2	Potassium nitrate	Reduction of nitrates to nitrites
TRP	Trytophan	Reduction of nitrates to nitrogen
GLU	Glucose	Acidification
ADH	Arginine	Presence of arginine dihydrolase
URE	Urea	Urease
ESC	Esculin	Hydrolysis
GEL	Gelatine	Hydrolsis
PNG	p-Nitrophenyl-β-D- galactopyranoside	β-galactosidase
GLU	Glucose	Assimilation
ARA	Arabinose	Assimilation
MNE	Mannose	Assimilation
MAN	Mannitol	Assimilation
NAG	N-Acetyl-glucosamine	Assimilation
MAL	Maltose	Assimilation
GNT	Gluconate	Assimilation
CAP	Caprate	Assimilation
ADI	Adipate	Assimilation
MLT	Malate	Assimilation
CIT	Citrate	Assimilation
PAC	Phenyl-acetate	Assimilation
OX	Tetramethyl-p- phenylene diamine	Cytochrome oxidase

API 50CH Carbohydrate codes

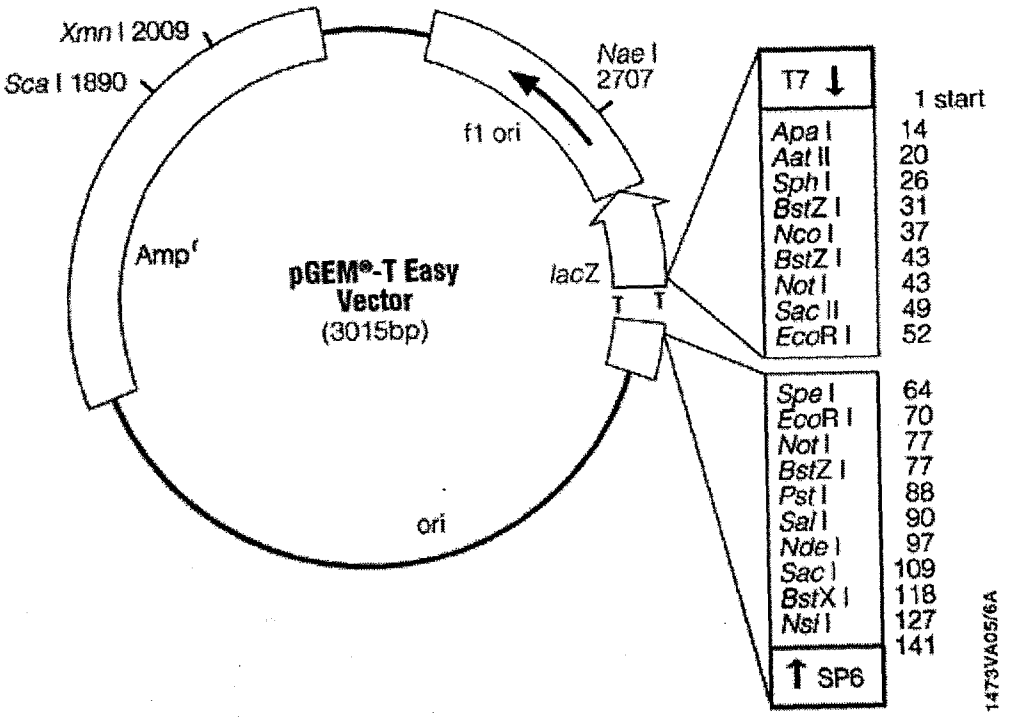
Test	Substrate
GLY	Glycerol
ERY	Erythirtol
D-ARA	D-Arabinose
L-ARA	L-Arabinose
RIB	Ribose
D-XYL	D- Xylose
L- XYL	L- Xylose
ADO	Adonitol
β-MDX	β-Methyl-D-xyloside
GAL	Galactose
GLU	Glucose
FRU	Fructose
MNE	Mannose
SBE	Sorbose
RHA	Rhamnose
DUL	Dulcitol
INO	Inositol
MAN	Mannitol
SOR	Sorbital
α-MDM	α-Methyl- D- mannoside
α-MDG	α-Methyl-D-glucoside
NAG	N-Acetyl- glucosamine
AMY	Amygdalin
ARB	Arbutin
ESC	Esculin
SAL	Salicin
CELI	Celiobiose
MAL	Maltose
LAC	Lactose
MEL	Melibiose
SAC	Sucrose
TRE	Trehalose
INU	Inulin

MLZ	Melezitose
RAF	Raffinose
STA	Starch
GLYG	Glycogen
XLT	Xylitol
GEN	Gentiobiose
DTUR	D-Turanose
DLYX	D-Lyxose
DTAG	D-Tagatose
DFUC	D-Fucose
DARL	D-Arabitol
LARL	L-Arabitol
GNT	Gluconate
2KG	2-Keto-gluconate
5KG	5-Keto-gluconate

A10 SEQUENCES OF PRIMERS USED FOR PCR AMPLIFICATIONS

PCR Name	Primer Name	Primer Sequence (5'-3')	Tm° C
Burkholderia **specific (O'Callaghan *et al.*, 1994)**	PC480	CTGTTCCGACCATTGTAT	56.1
	PC1240	GGTACCGGAAGAATAAGC	55.6
BOX Element (Martin *et al.*, 1992)	BOXAIR	CTACGGCAAGGCGACGCTGACG	76.5
ERIC (Hulton *et al.*, 1991)	ERIC1R	ATGTAAGCACCTGGGGATTCA	66
	ERIC 2	AAGTAAGTGACTGGGGTGAGC	68
Cable A gene (Sajjan *et al.*, 1995)	CblAF	CCAAAGGACTAACCCA	53.8
	CblAR	ACGCGATGTCCATCACA	62.6
16S rRNA gene sequencing (Yabuuchi *et al.*, 1992)	27F	GAGAGTTTGATCCTGGCTCAG	
	PC1240	GGTACCGGAAGAATAAGC	
Sequencing primers for PGEM-T Easy inserts	M13F	GTAAAACGACGGCCAGT	
	M13R	CAGGAAACAGCTATGAC	
B. cepacia **complex *recA* gene sequencing (Mahenthiralingam *et al.*, 2000)**	BCR1	TGACCGCCGAGAAGAGCAA	69.5
	BCR2	CTCTTCTTCGTCCATCGCCTC	67.5
RecA **gene sequencing**	Rec1R	TCTTCATCAACCAGMT	47.8
	Rec2R	GTAGCTGTACCAGGC	48.1
	Rec3R	CCTTGCCCTGGCCGAT	67.5
	Rec4F	GARGGCGARATGGGC	58.2
	Rec5F	TCSGGCAAGACCACS	52.2
	Rec6F	CTGGACTCGCVCTS	51.8
	Rec7F	GACCGCCGAGAAGAGCAA	66.7
	Rec8R	GCCGCGACCGAGTCGAT	70.2
Diagnostic *recA* gene primers – *B. fungorum*	FunF	GTCATGCGGCTCGGCGCAGGT	79.4
	FunR	GAGTGCGTCGGCAATCTCGAGT	71.8
Diagnostic *recA* gene primers- *B. sacchari*	SacF	GCCTCGAATCGCGCTCGGCGTG	81.7
	SacR	CCGAGCGACCCAGCGCGTCA	80.0
Diagnostic *recA* gene primers – CWP-01	CWP-01F	CCGAGAAGAGCAAGGCGCTGT	71.9
	CWP-01R	CGATCTTCGCCTGCACGCCC	76.2
Diagnostic *recA* gene primers –YWS-01	YWS-01F	CTCGTGCCGAAGGCCGTGATT	74.3
nifK **gene amplification (Minerdi *et al.*, 2001)**	NifKF	AAGGCTGCGTCGCCTATTACCG	71.6
	NifKR	GTCGCGCTCCAGATATTTGCC	69.7

A11 DIAGRAMMATIC REPRESENTATION OF pGEMT-EASY VECTOR (Taken from Promega website http://www.euro.promega.com/uk)



A12 COMBINED DATA FROM BOX, ERIC AND BOX-ERIC DENDROGRAMS

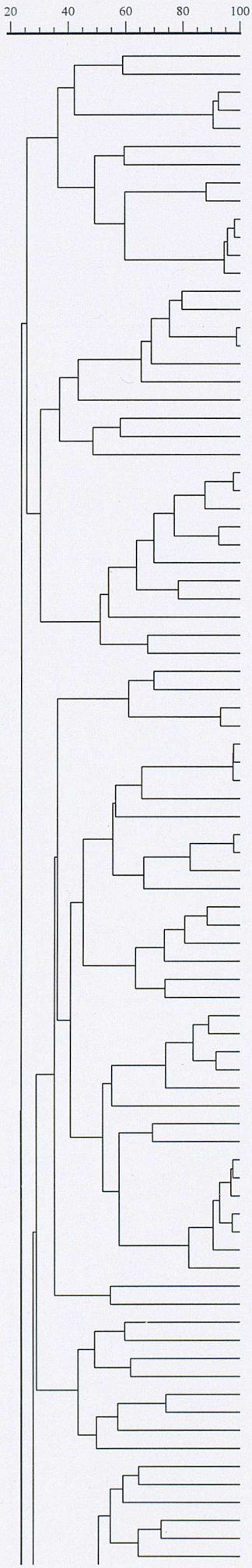
Combined BOX DNA fingerprint dendrogram (Jane Richardson and Chun-Han Chan)

Cophenetic correlation of 67.6%

(The asterisks * used to distinguish between novel isolates from the isolates of Jane Richardson.)

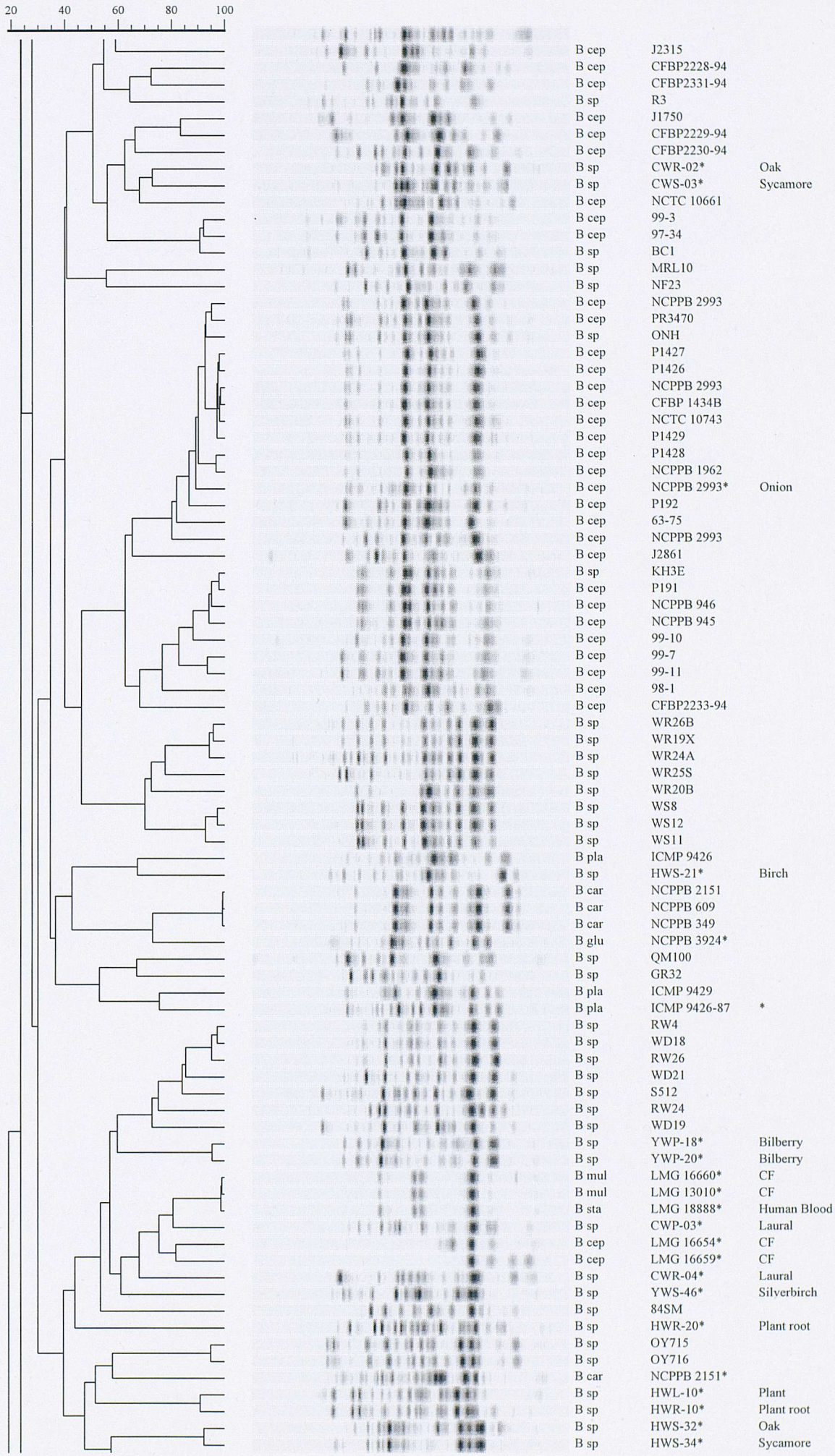


CJBOX (256 entries) Correlation - Fine [5-494] UPGMA



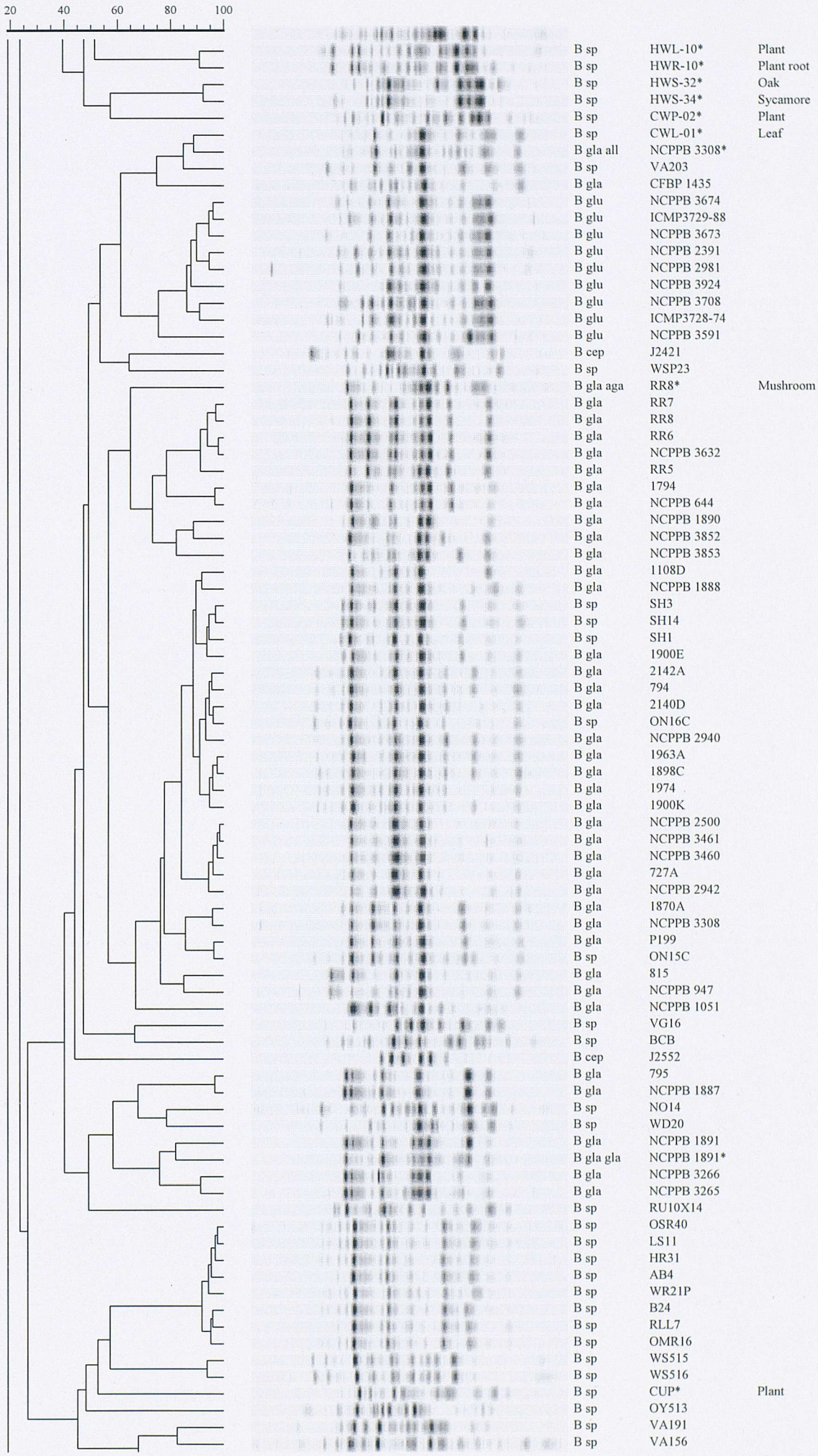
B cep	J1680	
B sp	WD16	
B sp	RW49	
B sp	RWS1	
B sp	RW48	
B sp	BCT	
B sac	LMG 19450*	Sugar cane
B cep	C1964	
B sp	A599	
B sp	CWS-01*	Oak
B sp	HWS-37*	Oak
B sp	HWS-44*	Oak
B sp	P13F*	Oak
B sp	VA144	
B sp	ON1	
B cal	LMG 19076*	Rhizosphere
B fun	LMG 16225*	Fungus
B sp	K14	
B cep	97-37	
B cep	LMG 18863*	CF
B sp	5N	
B sp	PHP64	
B sp	PHIG3	
B sp	VA157	
B sp	VA206	
B sp	406	
B sp	WR22B	
B sp	WR23S	
B sp	TW6	
B vie	LMG 10929*	Rhizosphere
B vie	LMG 18836*	CGD patient
B sp	WS16	
B sp	765	
B cep	NCPPB 3025	
B cep	CFBP2232-94	
B cep	CFBP 2232-94	* Clinical
B sp	NO10	
B sp	OY4F	
B gra	C4DIM*	Maize
B gra	C5AIM*	Maize
B gra	C3AIM*	Maize
B sp	SH13E	
B sp	31SM	
B sp	HWS-01*	Willow
B sp	HWS-04*	Beech
B sp	CWR-01*	Oak
B sp	CWR-03*	Sycamore
B sp	MU13	
B sp	13SM	
B sp	MU23	
B sp	10SM	
B sp	BRS55	
B sp	WW34	
B sp	RW10X8	
B sp	RW36	
B sp	MU2	
B sp	MU6	
B sp	CWP-01*	Laural
B sp	YWP-22*	Bilberry
B sp	WRP25	
B sp	GR34	
B and	NCPPB 1130	
B and	NCPPB 1127	
B and	NCPPB 934	
B and	NCPPB 1012	
B and	NCPPB 1013	
B and	NCPPB 1024	
B and	NCPPB 934*	
B sp	BCF/HG1	
B sp	OY714	
B sp	WR36	
B sp	HWR-15*	Plant root
B sp	STW1	
B sp	CWR-06*	Ash
B sp	CWS-02*	Oak
B sp	HWS-25*	Birch
B amb	LMG 19182*	Rhizosphere
B sp	K20	
B cep	NCPPB 3480	
B cep	NCTC10744	
B cep	J2315	
B cep	CFBP2228-94	
B cep	CFBP2331-94	
B sp	R3	

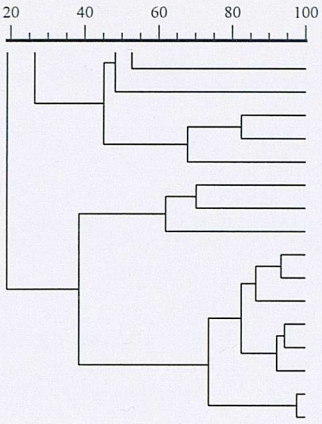
CJBOX (256 entries) Correlation - Fine [5-494] UPGMA

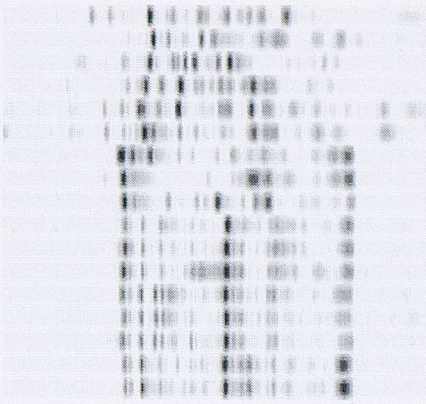


B cep	J2315	
B cep	CFBP2228-94	
B cep	CFBP2331-94	
B sp	R3	
B cep	J1750	
B cep	CFBP2229-94	
B cep	CFBP2230-94	
B sp	CWR-02*	Oak
B sp	CWS-03*	Sycamore
B cep	NCTC 10661	
B cep	99-3	
B cep	97-34	
B sp	BC1	
B sp	MRL10	
B sp	NF23	
B cep	NCPPB 2993	
B cep	PR3470	
B sp	ONH	
B cep	P1427	
B cep	P1426	
B cep	NCPPB 2993	
B cep	CFBP 1434B	
B cep	NCTC 10743	
B cep	P1429	
B cep	P1428	
B cep	NCPPB 1962	
B cep	NCPPB 2993*	Onion
B cep	P192	
B cep	63-75	
B cep	NCPPB 2993	
B cep	J2861	
B sp	KH3E	
B cep	P191	
B cep	NCPPB 946	
B cep	NCPPB 945	
B cep	99-10	
B cep	99-7	
B cep	99-11	
B cep	98-1	
B cep	CFBP2233-94	
B sp	WR26B	
B sp	WR19X	
B sp	WR24A	
B sp	WR25S	
B sp	WR20B	
B sp	WS8	
B sp	WS12	
B sp	WS11	
B pla	ICMP 9426	
B sp	HWS-21*	Birch
B car	NCPPB 2151	
B car	NCPPB 609	
B car	NCPPB 349	
B glu	NCPPB 3924*	
B sp	QM100	
B sp	GR32	
B pla	ICMP 9429	
B pla	ICMP 9426-87	*
B sp	RW4	
B sp	WD18	
B sp	RW26	
B sp	WD21	
B sp	S512	
B sp	RW24	
B sp	WD19	
B sp	YWP-18*	Bilberry
B sp	YWP-20*	Bilberry
B mul	LMG 16660*	CF
B mul	LMG 13010*	CF
B sta	LMG 18888*	Human Blood
B sp	CWP-03*	Laural
B cep	LMG 16654*	CF
B cep	LMG 16659*	CF
B sp	CWR-04*	Laural
B sp	YWS-46*	Silverbirch
B sp	84SM	
B sp	HWR-20*	Plant root
B sp	OY715	
B sp	OY716	
B car	NCPPB 2151*	
B sp	HWL-10*	Plant
B sp	HWR-10*	Plant root
B sp	HWS-32*	Oak
B sp	HWS-34*	Sycamore

CJBOX (256 entries) Correlation - Fine [5-494] UPGMA







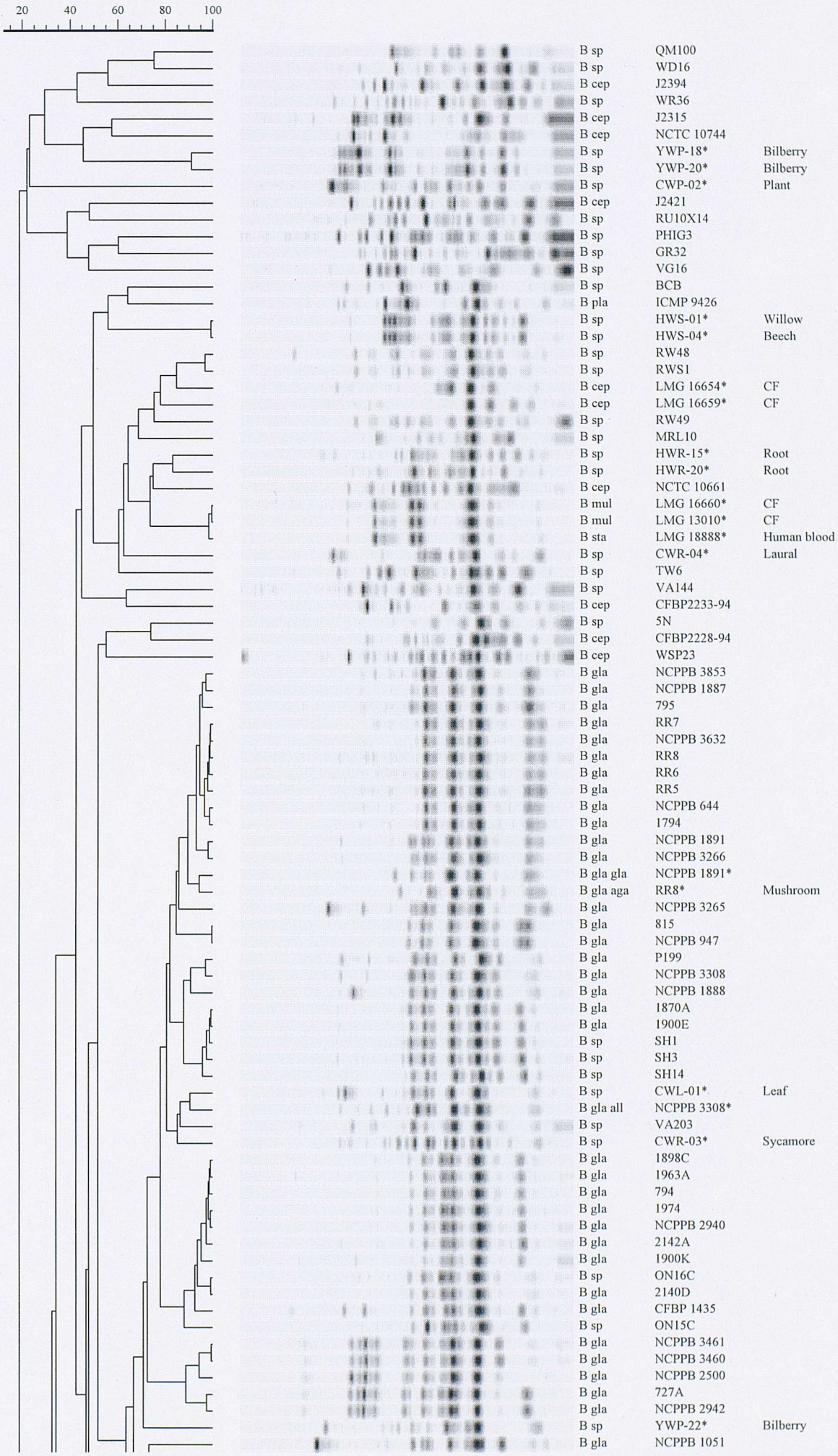
B sp	CUP*	Plant
B sp	OY513	
B sp	VA191	
B sp	VA156	
B sp	BC2	
B cep	C1576	
B cep	C1524	
B cep	J2394	
B sp	YWP-19*	Bilberry
B sp	YWP-21*	Bilberry
B sp	YWS-05*	Ash
B sp	YWS-48*	Silverbirch
B sp	YWS-49*	Silverbirch
B sp	YWS-47*	Silverbirch
B sp	HWS-27*	Woodland
B sp	HWS-29*	Beech

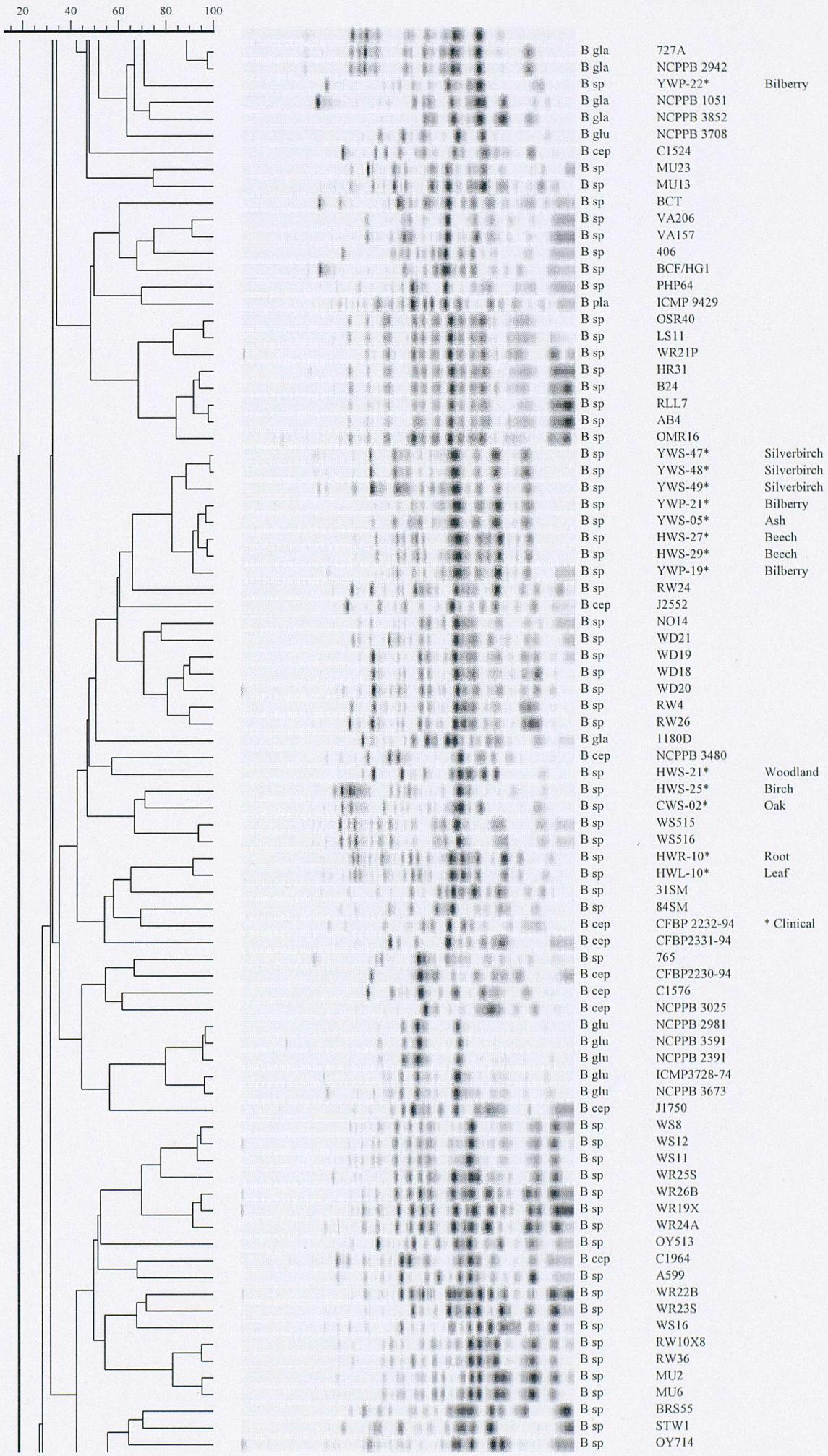
Combined ERIC DNA fingerprint dendrogram (Jane Richardson and Chun-Han Chan)

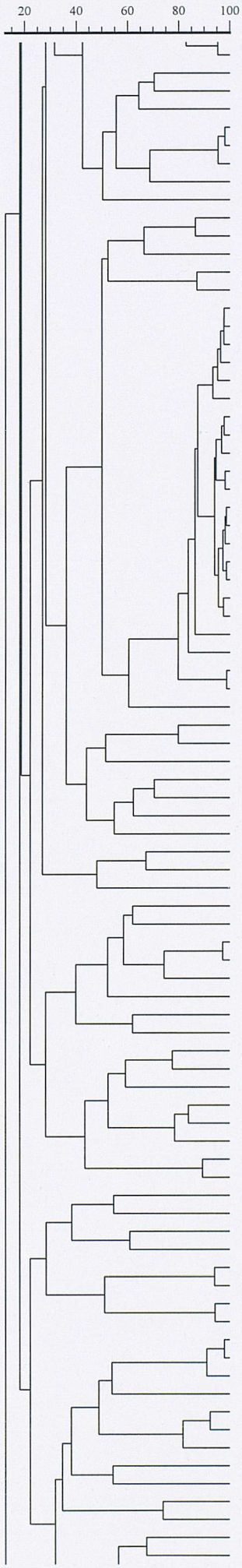
Cophenetic correlation of 72.3%

(The asterisks * used to distinguish between the novel isolates from the isolates of Jane Richardson.)

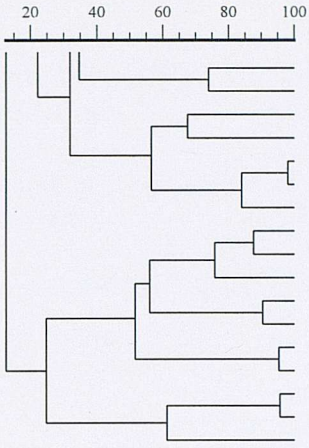



20
40
60
80
100
B sp QM100
B sp WD16
B cep J2394
B sp WR36
B cep J2315
B cep NCTC 10744
B sp YWP-18* Bilberry
B sp YWP-20* Bilberry
B sp CWP-02* Plant
B cep J2421
B sp RU10X14
B sp PHIG3
B sp GR32
B sp VG16
B sp BCB
B pla ICMP 9426
B sp HWS-01* Willow
B sp HWS-04* Beech
B sp RW48
B sp RWS1
B cep LMG 16654* CF
B cep LMG 16659* CF
B sp RW49
B sp MRL10
B sp HWR-15* Root
B sp HWR-20* Root
B cep NCTC 10661
B mul LMG 16660* CF
B mul LMG 13010* CF
B sta LMG 18888* Human blood
B sp CWR-04* Laural
B sp TW6
B sp VA144
B cep CFBP2233-94
B sp 5N
B cep CFBP2228-94
B cep WSP23
B gla NCPPB 3853
B gla NCPPB 1887
B gla 795
B gla RR7
B gla NCPPB 3632
B gla RR8
B gla RR6
B gla RR5
B gla NCPPB 644
B gla 1794
B gla NCPPB 1891
B gla NCPPB 3266
B gla gla NCPPB 1891*
B gla aga RR8* Mushroom
B gla NCPPB 3265
B gla 815
B gla NCPPB 947
B gla P199
B gla NCPPB 3308
B gla NCPPB 1888
B gla 1870A
B gla 1900E
B sp SH1
B sp SH3
B sp SH14
B sp CWL-01* Leaf
B gla all NCPPB 3308*
B sp VA203
B sp CWR-03* Sycamore
B gla 1898C
B gla 1963A
B gla 794
B gla 1974
B gla NCPPB 2940
B gla 2142A
B gla 1900K
B sp ON16C
B gla 2140D
B gla CFBP 1435
B sp ON15C
B gla NCPPB 3461
B gla NCPPB 3460
B gla NCPPB 2500
B gla 727A
B gla NCPPB 2942
B sp YWP-22* Bilberry
B gla NCPPB 1051


20
40
60
80
100
B gla 727A
B gla NCPPB 2942
B sp YWP-22* Bilberry
B gla NCPPB 1051
B gla NCPPB 3852
B glu NCPPB 3708
B cep C1524
B sp MU23
B sp MU13
B sp BCT
B sp VA206
B sp VA157
B sp 406
B sp BCF/HG1
B sp PHP64
B pla ICMP 9429
B sp OSR40
B sp LS11
B sp WR21P
B sp HR31
B sp B24
B sp RLL7
B sp AB4
B sp OMR16
B sp YWS-47* Silverbirch
B sp YWS-48* Silverbirch
B sp YWS-49* Silverbirch
B sp YWP-21* Bilberry
B sp YWS-05* Ash
B sp HWS-27* Beech
B sp HWS-29* Beech
B sp YWP-19* Bilberry
B sp RW24
B cep J2552
B sp NO14
B sp WD21
B sp WD19
B sp WD18
B sp WD20
B sp RW4
B sp RW26
B gla 1180D
B cep NCPPB 3480
B sp HWS-21* Woodland
B sp HWS-25* Birch
B sp CWS-02* Oak
B sp WS515
B sp WS516
B sp HWR-10* Root
B sp HWL-10* Leaf
B sp 31SM
B sp 84SM
B cep CFBP 2232-94 * Clinical
B cep CFBP2331-94
B sp 765
B cep CFBP2230-94
B cep C1576
B cep NCPPB 3025
B glu NCPPB 2981
B glu NCPPB 3591
B glu NCPPB 2391
B glu ICMP3728-74
B glu NCPPB 3673
B cep J1750
B sp WS8
B sp WS12
B sp WS11
B sp WR25S
B sp WR26B
B sp WR19X
B sp WR24A
B sp OY513
B cep C1964
B sp A599
B sp WR22B
B sp WR23S
B sp WS16
B sp RW10X8
B sp RW36
B sp MU2
B sp MU6
B sp BRS55
B sp STW1
B sp OY714



B sp	MU6	
B sp	BRS55	
B sp	STW1	
B sp	OY714	
B car	NCPPB 349	
B car	NCPPB 2151	
B car	NCPPB 609	
B cep	97-37	
B sp	ON1	
B sp	CWR-06*	Ash
B sp	CWS-03*	Sycamore
B sp	CWS-01*	Oak
B sp	NO10	
B sp	OY4F	
B sp	KH3E	
B cep	99-10	
B cep	98-1	
B cep	NCPPB 945	
B cep	NCPPB 946	
B cep	P191	
B cep	NCPPB 2993	
B cep	PR3470	
B sp	ONH	
B cep	P192	
B cep	NCPPB 1962	
B cep	P1426	
B cep	P1427	
B cep	P1429	
B cep	NCPPB 2993	
B cep	P1428	
B cep	CFBP 1434B	
B cep	NCTC 10743	
B cep	NCPPB 2993*	Onion
B cep	NCPPB 2993	
B cep	99-7	
B cep	99-11	
B cep	63-75	
B sp	13SM	
B sp	10SM	
B cep	J2861	
B sp	CWR-01*	Oak
B sp	YWS-46*	Silverbirch
B sp	S512	
B sp	SH13E	
B gla	NCPPB 1890	
B cep	LMG 18863*	CF
B sp	WR20B	
B sp	K14	
B gra	C5AIM*	Maize
B gra	C3AIM*	Maize
B gra	C4DIM*	Maize
B pla	ICMP 9429-87	*
B car	NCPPB 2151*	
B cep	CFBP2229-94	
B sp	WW34	
B vie	LMG 10929*	Rhizosphere
B vie	LMG 18836*	Rhizosphere
B sp	GR34	
B sp	VA191	
B sp	VA156	
B sp	BC2	
B cep	CFBP2232-94	
B cep	CFBP2232-94	
B sp	NF23	
B sac	LMG 19450*	Sugar cane
B sp	CUP*	Plant
B and	NCPPB 934*	
B sp	OY715	
B sp	OY716	
B glu	ICMP3729-88	
B glu	NCPPB 3674	
B and	NCPPB 934	
B and	NCPPB 1013	
B and	NCPPB 1012	
B cep	J1680	
B and	NCPPB 1130	
B and	NCPPB 1127	
B and	NCPPB 1024	
B sp	K20	
B sp	WRP25	
B glu	NCPPB 3924	
B glu	NCPPB 3924*	
B sp	R3	
B sp	CWP-03*	Laural



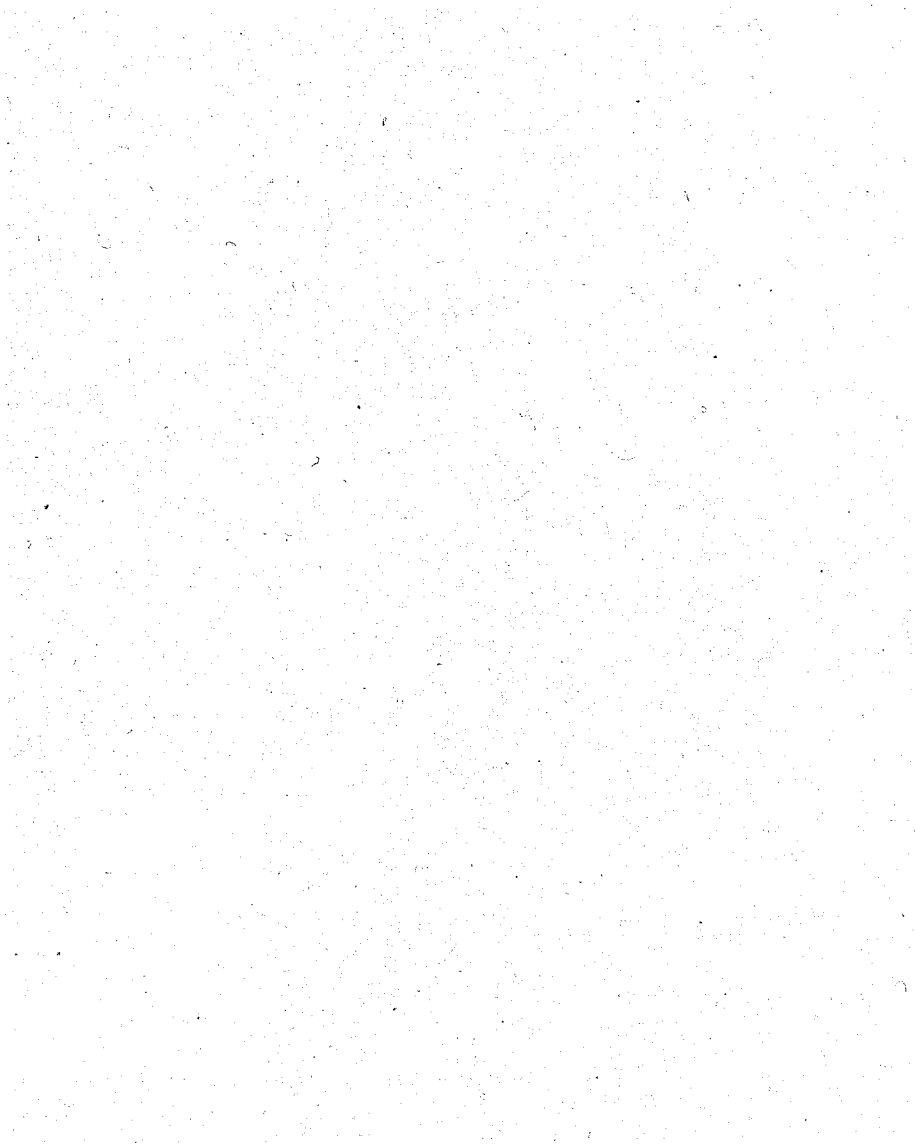


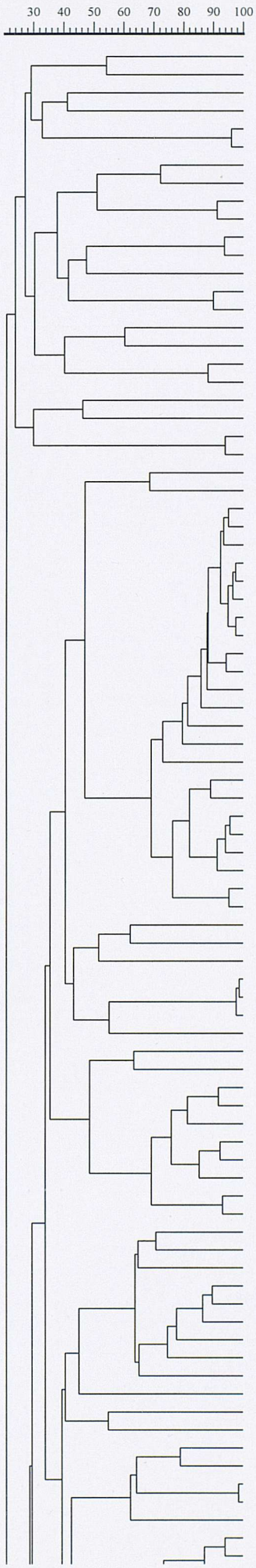
B glu	NCPPB 3924	
B glu	NCPPB 3924*	
B sp	R3	
B sp	CWP-03*	Laural
B cep	97-34	
B cep	99-3	
B sp	BC1	
B sp	HWS-44*	Oak
B sp	P13F*	Oak
B sp	HWS-37*	Oak
B sp	HWS-32*	Oak
B sp	HWS-34*	Sycamore
B sp	CWP-01*	Laural
B sp	CWR-02*	Oak
B cal	LMG 19076*	Rhizosphere
B fun	LMG 16225*	Fungus
B amb	LMG 19182*	Rhizosphere

Combined BOX-ERIC DNA fingerprint dendrogram (Jane Richardson and Chun-Han Chan)

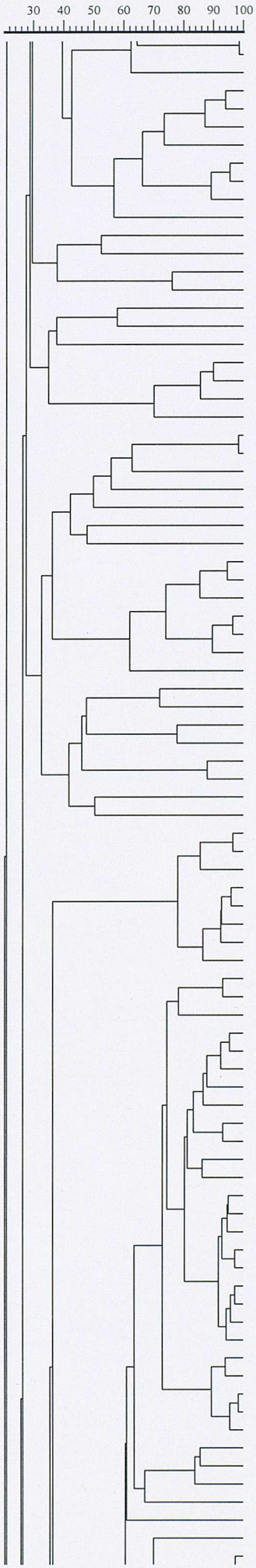
Cophenetic correlation of 76.8%

(The asterisks * used to distinguish between the novel isolates from the isolates of Jane Richardson.)

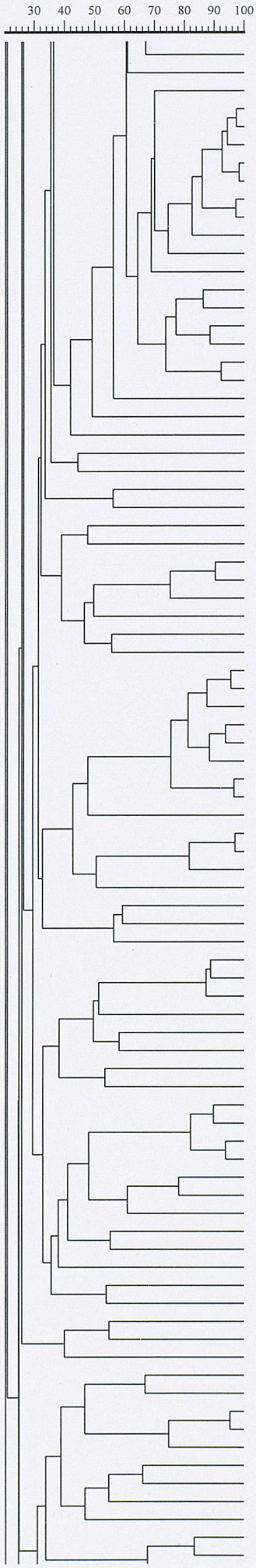




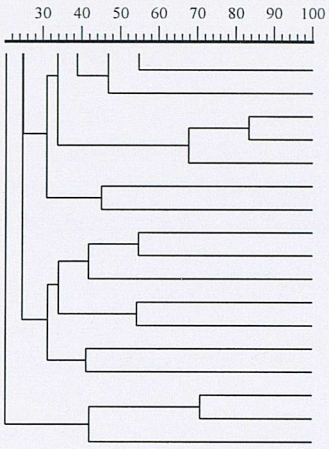
B sp	CWP-01*	Laural
B sp	CWR-02*	Oak
B sp	K20	
B amb	LMG 19182*	Rhizosphere
B cal	LMG 19076*	Rhizosphere
B fun	LMG 16225*	Fungus
B sp	CWS-02*	Oak
B sp	HWS-25*	Birch
B sp	HWS-32*	Oak
B sp	HWS-34*	Sycamore
B sp	WS515	
B sp	WS516	
B sp	CUP*	Plant
B sp	HWL-10*	Plant
B sp	HWR-10*	Plant root
B sp	31SM	
B mul	LMG 16660*	CF
B sp	YWP-18*	Bilberry
B sp	YWP-20*	Bilberry
B sp	NF23	
B sac	LMG 19450*	Sugar cane
B sp	OY716	
B sp	OY715	
B sp	CWR-06*	Ash
B sp	CWS-03*	Sycamore
B sp	ONH	
B cep	PR3470	
B cep	NCPPB 2993	
B cep	CFBP 1434B	
B cep	NCTC 10743	
B cep	P1429	
B cep	NCPPB 2993	
B cep	P1426	
B cep	P1428	
B cep	NCPPB 1962	
B cep	P192	
B cep	NCPPB 2993*	Onion
B cep	P1427	
B cep	NCPPB 2993	
B cep	63-75	
B cep	99-10	
B cep	98-1	
B sp	KH3E	
B cep	NCPPB 945	
B cep	NCPPB 946	
B cep	P191	
B cep	99-7	
B cep	99-11	
B cep	NCTC 10661	
B cep	CFBP2228-94	
B sp	MRL10	
B car	NCPPB 349	
B car	NCPPB 2151	
B car	NCPPB 609	
B cep	97-37	
B glu	NCPPB 3924	
B glu	NCPPB 3924*	
B glu	NCPPB 3673	
B glu	ICMP3728-74	
B glu	NCPPB 3708	
B glu	NCPPB 2391	
B glu	NCPPB 2981	
B glu	NCPPB 3591	
B glu	NCPPB 3674	
B glu	ICMP3729-88	
B sp	NO14	
B sp	WD20	
B sp	S512	
B sp	RW4	
B sp	WD18	
B sp	RW26	
B sp	WD19	
B sp	WD21	
B sp	RW24	
B sp	84SM	
B cep	J2861	
B sp	YWS-46*	Silverbirch
B cep	LMG 16654*	CF
B cep	LMG 16659*	CF
B sta	LMG 18888*	Human blood
B mul	LMG 13010*	CF
B sp	CWR-04*	Laural
B sp	WR24A	
B sp	WR26B	



B mul	LMG 13010*	CF
B sp	CWR-04*	Laural
B sp	WR24A	
B sp	WR26B	
B sp	WR19X	
B sp	WR25S	
B sp	WS12	
B sp	WS8	
B sp	WS11	
B sp	WR20B	
B sp	R3	
B sp	CWP-03*	Laural
B sp	C1964	
B sp	A599	
B cep	CFBP2232-94	
B cep	CFBP2232-94	* Clinical
B sp	CWP-02*	Plant
B sp	HWS-44*	Oak
B sp	P13F*	Oak
B sp	HWS-37*	Oak
B sp	CWS-01*	Oak
B sp	HWS-01*	Woodland
B sp	HWS-04*	Beech
B sp	CWR-01*	Oak
B sp	YWP-22*	Bilberry
B cep	CFBP2233-94	
B sp	GR34	
B sp	WRP25	
B and	NCPPB 1130	
B and	NCPPB 1127	
B and	NCPPB 1024	
B and	NCPPB 1013	
B and	NCPPB 934	
B and	NCPPB 1012	
B and	NCPPB 934*	
B sp	MU13	
B sp	MU23	
B sp	10SM	
B sp	13SM	
B sp	NO10	
B sp	OY4F	
B sp	SH13E	
B sp	WW34	
B sp	OSR40	
B sp	LS11	
B sp	WR21P	
B sp	RLL7	
B sp	AB4	
B sp	B24	
B sp	HR31	
B sp	OMR16	
B gla	815	
B gla	NCPPB 947	
B gla	CFBP 1435	
B gla	1900E	
B sp	SH3	
B sp	SH1	
B sp	SH14	
B gla	NCPPB 1888	
B gla	1870A	
B gla all	NCPPB 3308	
B gla	P199	
B sp	ON15C	
B gla	794	
B gla	NCPPB 2940	
B gla	2142A	
B sp	ON16C	
B gla	2140D	
B gla	1898C	
B gla	1963A	
B gla	1974	
B gla	1900K	
B gla	727A	
B gla	NCPPB 2942	
B gla	NCPPB 3461	
B gla	NCPPB 3460	
B gla	NCPPB 2500	
B sp	VA203	
B sp	CWL-01*	Leaf
B gla all	NCPPB 3308*	
B sp	ON15C	
B gla	NCPPB 1051	
B gla aga	RR8*	Mushroom
B gla	RR7	



B sp	ON15C	
B gla	NCPPB 1051	
B gla aga	RR8*	Mushroom
B gla	RR7	
B gla gla	RR8	
B gla	RR5	
B gla	RR6	
B gla	NCPPB 3632	
B gla	NCPPB 644	
B gla	1794	
B gla	NCPPB 3853	
B gla	NCPPB 3852	
B gla	NCPPB 1890	
B gla gla	NCPPB 1891	
B gla gla	NCPPB 1891*	
B gla	NCPPB 3266	
B gla	NCPPB 3265	
B gla	795	
B gla	NCPPB 1887	
B gla	1108D	
B sp	CWR-03*	Sycamore
B sp	WSP23	
B cep	J2421	
B cep	J2552	
B pla	ICMP9426-87	
B sp	HWS-21*	Birch
B sp	BCF/HG1	
B sp	BCT	
B sp	VA206	
B sp	VA157	
B sp	406	
B sp	BCB	
B sp	PHP64	
B cep	CFBP2331-94	
B sp	YWS-47*	Silverbirch
B sp	YWS-48*	Silverbirch
B sp	YWS-49*	Silverbirch
B sp	YWP-21*	Bilberry
B sp	YWP-19*	Bilberry
B sp	YWS-05*	Ash
B sp	HWS-27*	Woodland
B sp	HWS-29*	Beech
B sp	OY513	
B gra	C3AIM*	Maize
B gra	C4DIM*	Maize
B gra	C5AIM*	Maize
B sp	K14	
B cep	C1576	
B cep	C1524	
B cep	J2394	
B sp	RW48	
B sp	RW49	
B sp	RWS1	
B sp	TW6	
B sp	HWR-15*	Plant root
B sp	HWR-20*	Plant root
B sp	VA144	
B sp	ON1	
B sp	RW36	
B sp	RW10X8	
B sp	MU6	
B sp	MU2	
B sp	WR23S	
B sp	WR22B	
B sp	WS16	
B sp	BRS55	
B sp	OY714	
B sp	WR36	
B sp	5N	
B sp	WD16	
B cep	NCPPB 3025	
B sp	765	
B sp	RU10X14	
B cep	J1750	
B cep	CFBP2229-94	
B cep	97-34	
B cep	99-3	
B sp	BC1	
B pla	ICMP 9429	
B pla	ICMP9426-87	*
B cep	CFBP2230-94	
B car	NCPPB 2151*	
B sp	VA156	
B sp	VA191	





B cep	CFBP2230-94	
B car	NCPPB 2151*	
B sp	VA156	
B sp	VA191	
B sp	BC2	
B sp	QM100	
B sp	GR32	
B cep	J1680	
B cep	NCPPB 3480	
B sp	STW1	
B cep	J2315	
B cep	NCTC10744	
B sp	VG16	
B sp	PHIG3	
B vie	LMG 10929*	Rhizosphere
B vie	LMG 18836*	CGD patient
B cep	LMG 18863*	CF

REFERENCES

Achouak, W., Christen, R., Barakat, M., Martel, M-H., and Heulin, T. (1999) *Burkholderia caribensis* sp. nov. an exopolysaccharide-producing bacterium isolated from vertisol microaggregates in Martinique. *International Journal of Systematic Bacteriology.* **49**, 787-794.

Adjei, M.D & Ohta, Y. (2000) Purification and properties of a cyanide- degrading enzyme from *Burkholderia cepacia* strain C-3. *World Journal of Microbiology & Biotechnology.* **16**, 171-75.

Adnan, S., Li, N., Miura, H., Hashimoto., Yamamoto, H & Ezaki, T. (1993) Covalently immobilized DNA plate for luminometric DNA-DNA hybridization to identify viridans streptococci in under 2 hours. *FEMS Microbiology Letters.* **106**, 139-42.

Balandreau, J., Viallard, V., Cournoyer, B., Coenye, T., Laevens, S & Vandamme, P. (2001). *Burkholderia cepacia* genomovar III Is a common plant-associated bacterium. *Applied Environmental Microbiology.* **67**(2), 982-5

Ballard, R.W., Palleroni, N.J., Doudoroff, M & Stanier, R.Y. (1970) Taxonomy of the aerobic Pseudomonads : *Pseudomonas cepacia*, *P.marginata*, *P. allicola* and *P.caroyophylli. Journal of General Microbiology.* **60**, 199-214.

Bevivino, A., Dalmastri, C., Tabacchioni, S., Chiarini, L., Belli, M.L., Piana, S., Materazzo, A., Vandamme, P & Manno, G (2002) *Burkholderia cepacia* complex bacteria from clinical and environmental sources in Italy: Genomovar status and distribution of traits related to virulence and transmissibility. *Journal of Clinical Microbiology.* **40** (3), 846-51.

Blocker, A., Holden, D & Cornellis, G. (2000) Type III secretion systems: what is the translocator and what is translocated. *Cellular Microbiology.* **2** (5), 387-90.

Bopp, L.H. (1986) Degradation of highly chlorinated PCB's by Pseudomonas strain LB400. *Journal of Industrial Microbiology.* **1**, 23-9

Bramer, C.O., Vandamme, P., da Silva, L.F., Gomez, J.G & Steinbuchel, A (2001) Polyhydroxyalkanoate-accumulating bacterium isolated from soil of a sugar-cane plantation in Brazil. *International Journal of Systematic and Evolutionary Microbiology.* **51** (5). 1709-13.

Brett, B & Waldron, K (1996) *Physiology and Biochemistry of Plant Cell Wall*, 2nd Edition. Chapman and Hall, London, United Kingdom.

Brett, P. J., DeShazer, D., and Woods, D. E. (1997) Characterization of *Burkholderia pseudomallei* and *Burkholderia pseudomallei* - like strains. *Epidemiology and Infections.* **118**, 137-148.

Brett, P. J., DeShazer, D., and Woods, D. E. (1998) *Burkholderia thailandensis* sp. nov., a *Burkholderia pseudomallei*-like species. *International Journal of Systematic Bacteriology.* **48**, 317-320.

Burbage, D.A & Sasser, M. (1982) A medium selective for *Pseudomonas cepacia. Phytopathology Abstracts.* **72**, 706.

Burkholder, W.H. (1950) Sour Skin: A bacterial rot of onion bulbs. *Phytopathology*. **40**, 115-7.

Butler, S.I., Doherty, C.J., Hughes, J.E., Nelson, J.W & Govan, J.R.W. (1995) *Burkholderia cepacia* and cystic fibrosis: Do natural environmental present a potential hazard? *Journal of Clinical Microbiology*. **33** (4), 1001-4.

Cain, C.C., Henry, A.T., Waldo III, R.H., Casida Jnr, L.J & Falkinham III, J.O. (2000) Identification and characteristics of a novel Burkholderia strain with broad-spectrum antimicrobial activity. *Applied and Environmental Microbiology*. **66** (9), 4139-41.

Cartwright, D.K., Chilton, W.S & Benson, D.M. (1995) Pyrrolnitrin and phenazine production by *Pseudomonas cepacia* strain 5.5B, a biocontrol agent of *Rhizoctonia solani*. *Applied Microbiology and Biotechnology,* **43**, 211-16.

Charlebois, R (Ed) Insertion sequences and transposons. In: *Organization of the prokaryotic genome*. American Society of Microbiology Press, Washington, USA.

Chen, J.S., Witzmann, K.A., Spilker, T., Fink, R.J & LiPuma, J.J. (2001) Endemicity and inter-city spread of *Burkholderia cepacia* genomovar III in cystic fibrosis. *Journal of Paediatrics*. **139** (5), 643-49.

Cheng, H.P & Lessie, T.G. (1994) Multiple replicons constituting the genome of *Pseudomonas cepacia* 17616. *Journal of Bacteriology*. **176** (13), 4034-42.

Chung, J.W., Altman, E., Beveridge, T.J & Speert, D.P. (2003) Colonial morphology of *Burkholderia cepacia* complex genomovar III: Implications in exopolysaccharide production, pilus expression, and persistence in the mouse. *Infection and Immunity*. **71** (2), 904-9.

Chiarini, L., Giovannelli, V., Bevivino, A., Dalmastri, C & Tabacchioni, S. (2000) Different portions of the maize root system host *Burkholderia cepacia* populations with different degrees of genetic polymorphism. *Environmental Microbiology*. **2** (1), 111-18.

Clark, A. J & Margulies, A.D. (1965) Isolation and characterization of recombination-deficient mutants of *Escherichia coli* K12. *Proceedings of the National Academy of Science, U.S.A*. **53**, 451-9.

Clerc, A., Manceau, C & Nesme, X. (1998) Comparison of randomly amplified polymorphic DNA with amplified fragment length polymorphism to assess genetic diversity and genetic relatedness within genospecies III of *Pseudomonas syringae*. *Applied and Environmental Microbiology*. **64** (4), 1180-87.

Clode, F.E., Kaufmann, M.E., Malnick, H & Pitt, T.L. (2000) Distribution of genes encoding putative transmissibility factors among epidemic and nonepidemic strains of *Burkholderia cepacia* from cystic fibrosis patients in the United Kingdom. *Journal of Clinical Microbiology*. **38** (5), 1763-66.

Coenye, T., Holmes, B., Kersters, K., Govan, J.R.W & Vandamme, P (1999a) *Burkholderia cocovenenans* (van Damme *et al.* 1960) Gillis *et al.* 1995 and *Burkholderia vandii* Urakami *et al.* 1994 are junior synonyms of *Burkholderia gladioli* (Severini 1913) Yabuuchi *et al.* 1993 and *Burkholderia plantarii* (Azegami *et al.* 1987) Urkami *et al.* 1994, respectively. *International Journal of Systematic Bacteriology*. **49**, 37-42.

Coenye, T., Schouls, L.M., Govan, J.R., Kersters, K & Vandamme P (1999b) Identification of *Burkholderia* species and genomovars from cystic fibrosis patients by AFLP fingerprinting. *International Journal of Systematic Bacteriology.* **49** (4), 1657-66

Coenye, T., Falsen, E., Hoste, B., Ohlen, M., Goris, J., Govan, J. R. W., *et al.* (2000) Description of *Pandoraea* gen. nov. with *Pandoraea apista* sp. nov., *Pandoraea pulmonicola* sp. nov., *Pandoraea pnomenusa* sp. nov., *Pandoraea sputorum* sp. nov. and *Pandoraea norimbergensis* comb. nov. *International Journal of Systematic and Evolutionary Microbiology.* **50**, 887-899.

Coenye, T, Laevens, S., Gillis, M & Vandamme, P (2001a) Genotypic and chemotaxonomic evidence for the reclassification of *Pseudomonas woodsii* (Smith 1911) Stevens 1925 as *Burkholderia andropogonis* (Smith 1991) Gillis *et al.* 1995. *International Journal of Systematic and Evolutionary Microbiology.* **51**, 183-5.

Coenye, T., LiPuma, J.J., Henry, D., Hoste, B., Vandemeulebroecke, K., Gillis, M., Speert, D. P & Vandamme, P. (2001b) *Burkholderia cepacia* genomovar VI, a member of the *Burkholderia cepacia* complex isolated from cystic fibrosis patients. *International Journal of Systematic and Evolutionary Microbiology.* **51**, 271-79.

Coenye, T., Laevens, S., Willems, A., Ohlen, M., Hannant, W., Govan, R.W., Gillis, M., Falsen, E & Vandamme, P. (2001c) *Burkholderia fungorum* sp. nov and *Burkholderia caledonica* sp. nov., two new species isolated from the environmental, animals and human clinical samples. *International Journal of Systematic and Evolutionary Microbiology.* **51**, 1099-107.

Coenye, T., Mahenthiralingam, E., Henry, D., LiPuma, J,J., Laevens, S., Gillis, M., Speert, D.P & Vandamme, P. (2001d) *Burkholderia ambifaria* sp. nov., a novel member of the *Burkholderia cepacia* complex including biocontrol and cystic fibrosis-related isolate. *International Journal of Systematic and Evolutionary Microbiology.* **51**, 1481-90.

Coenye,T., Vandamme, P., Govan, J.R.W & LiPuma J.J (2001e) Taxonomy and identification of the *Burkholderia cepacia* complex. *Journal of Clinical Microbiology.* **39** (10), 3427-36.

Coenye, T & LiPuma, J.J (2002) Population structure analysis of *Burkholderia cepacia* genomovar III: varying degrees of genetic recombination characterize major clonal complexes. *Microbiology.* **149**, 77-88.

Coenye, T., Spilker, T., Martin, & LiPuma, J.J. (2002) Comparative assessment of genotyping methods for epidemiologic study of *Burkholderia cepacia* genomovar III. *Journal of Clinical Microbiology.* **40** (9), 3300-07.

Dalmastri, C., Chiarini, L., Cantale, C., Bevivino, A & Tabacchioni, S (1999) Soil Type and Maize Cultivar Affect the Genetic Diversity of Maize Root-Associated *Burkholderia cepacia* Populations. *Microbial Ecology.* **38** (3), 273-284.

De Ley, J., Cattoir, H & Reynaerts, A. (1970) The quantitative measurement of DNA hybridisation from renaturation rates. *European Journal of Biochemistry.* **12**, 133-42.

Devereaux, J., Haeberli, P & Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research.* **12**, 387-95.

Di Cello, F., Bevivino, A., Chiarini, L., Fani, R., Paffetti, D., Tabacchioni, S & Dalmastri, C. (1997) Biodiversity of a *Burkholderia cepacia* population isolated from the maize rhizosphere at different plant growth stages. *Applied and Environmental Microbiology.* **63**, 4485-4493.

Eisen, J.A. (1995) The *recA* protein as a model molecule for molecular systematic studies of bacteria: comparison of trees of *recA*s and 16S rRNA from the same species. *Journal of Molecular Evolution.* **41** (6), 1105-23.

Esiobu, N., Armenta, L & Ike, J. (2002) Antibiotic resistance in soil and water environments. *International Journal of Environmental Health Research.* **12** (2), 133-44.

Estrada-De-Los Santos, P., Bustillos-Christales, R & Caballero-Mellado, J. (2001) *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. *Applied and Environmental Microbiology.* **67** (6), 2790-98.

Ezaki, T., Hasimoto, Y & Yabuuchi, E. (1989) Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridisation in microdilution wells as an alternative to membrane filter hybridisation in which radioisotopes are used to determine genetic relatedness among bacterial strains. *International Journal of Systematic Bacteriology.* **39** (3), 224-29.

Fain, M. G., and Haddock, J. D. (2001) Phenotypic and phylogenetic characterisation of *Burkholderia* (*Pseudomonas*) sp. strain LB400. *Current Microbiology.* **4**, 269-275.

Feinberg, A.P & Vogelstein, B (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry.* **132** (1), 6-13.

Fiore, A., Laevens, S., Bevivino, A., Dalmastri, C., Tacbacchioni, S., Vandamme, P & Chiarini, L. (2001) *Burkholderia cepacia* complex: distribution of genomovars among isolates from the maize rhizosphere in Italy. *Environmental Microbiology.* **3** (2), 137-43.

Forster, A.C., McInnes, J., Skingle, D.C & Symons, R.H (1985) Non-radioactive probes prepared by the chemical labelling of DNA and RNA with a novel reagent, photobiotin. *Nucleic Acids Research.* **13** (3), 745-61.

Friedrich, M., Grosser, R. J., Kern, E. A., Inskeep, W. P., and Ward, D. M. (2000) Effect of model sorptive phases on phenanthrene biodegradation: molecular analysis of enrichments and isolates suggests selection based on bioavailability. *Applied and Environmental Microbiology.* **66**, 2703-2710.

Geiger, A.M., Hogardt, M & Hessemann, J. (2001) *Burkholderia / Stenotrophomonas. Emerging Bacterial Pathogens.* **8**, 20-34.

Gillis, M., Van, T.V., Bardin, R., Goor, M., Hebbar, P., Willems, A., Segers, P., Kersters, K., Heulin, T & Fernandez, M.P (1995) Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp nov. for N2- fixing isolates from rice in Vietnam. *International Journal of Systemic Bacteriology.* **45**, 274-89.

Goldmann, D.A & Klinger, M.P.H (1986) *Pseudomonas cepacia*: biology, mechanisms of virulence, epidemiology. *Journal of Pediatrics*. **5** (2), 806-12.

Goldstein, R., Sun, L., Jiang, R.Z., Sajjan, U., Forstner, J.F & Campanelli, C. (1995) Structurally variant classes of pilus appendage fibers coexpressed from *Burkholderia* (*Pseudomonas*) *cepacia*. *Journal of Bacteriology*. **177** (4), 1039-52.

Goris, J., Dejonghe, W., Falsen, E., De Clerck, E., Geeraerts, B., Willems, A., Top, E.M, Vandamme, P & De Vos, P. (2002) Diversity of transconjugants that acquired plasmid pJP4 or pEMT1 after inoculation of a donor strain in the A- and B- horizon of an agricultural soil and description of *Burkholderia hospita* sp. nov. and *Burkholderia terricola* sp. nov. *Systematic and Applied Microbiology*. **25**, 340-52.

Govan, J.R & Harris, G. (1985) Typing of *Pseudomonas cepacia* by bacteriocin susceptibility and production. *Journal of Clinical Microbiology*. **4**, 490-4

Govan, J.R.W & Deretic, V. (1996) Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiological Reviews*. **60** (3), 539-74.

Govan, J.R., Hughes, J.E & Vandamme, P (1996) *Burkholderia cepacia*: medical, taxonomic and ecological issues. *Journal of Medical Microbiology*. **45** (6), 395-407.

Govan, J.R.W & Vandamme, P. (1998) Agricultural and medical microbiology: a time for bridging gaps. *Microbiology*. **144** (9), 2373-75.

Groisman, E.A & Ochman, H. (1996) Pathogenicity islands: bacterial evolution in quantum leaps. *Cell*. **87**, 791-4.

Gross, D.C & Cody. Y.S (1985) Mechanisms of plant pathogenesis by Pseudomonas species. *Canadian Journal of Microbiology*. **34**, 403-10.

Guttman, D.S & Dykhuizen, D.E (1994) Clonal divergence in *Escherichia coli* as a result of recombination not mutation. *Science*. **266**, 1380-3.

Hacker, J., Blum-Oehler, G., Mühldorfer, I & Tschäpe, H (1997) Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Molecular Microbiology*. **23** (6), 1089-97.

Hagedorn, C., Gould, W.D., Bardinelli, T.R & Gustavson, D.R (1987) A selective medium for enumeration and recovery of *Pseudomonas cepacia* biotypes from soil. *Applied and Environmental Microbiology*. **53** (9), 2265-68

Hales, B.A., Moogan, W.A., Hart, C.A & Winstanley, C. (1998) Variation in flagellin genes and proteins of *Burkholderia cepacia*. *Journal of Bacteriology*. **180** (5), 1110-8.

Hanada, S., Shigematsu, T., Shibuya, K., Eguchi, M., Hasegawa, T., Suda, *et al.* (1998) Phylogenetic analysis of trichloroethylene-degrading strains newly isolated from the polluted soil with the contaminant. *Journal of Fermentation and Bioengineering*. **86**, 539-544.

Hayatsu, M., Hirano, M & Tokuda, S. (2000) Involvement of two plasmids in fenitrothion degradation by *Burkholderia* sp. strain NF100. *Applied and Environmental Microbiology*. **66** (4), 1737-40.

Hebbar, K.P., Davey., A.G., Merrin, J., M^{c}Loughlin, T.J & Dart, P.J. (1992) *Pseudomonas cepacia*, a potential supressor of maize soil-borne diseases – seed inoculation and maize root colonization. *Soil Biology and Biochemistry*, **24** (10), 999-1007.

Holmes, A., Govan, J & Goldstein, R. (1998) Agricultural use of *Burkholderia* (*Pseudomonas*) *cepacia*. *Emerging Infectious Diseases*. **4** (2), 221-7.

Hou, Y.M. (1999) Transfer RNAs and pathogenicity islands. *Trends In Biochemical Science*. **24**, 295-98.

Hulton, C.S.J., Higgins, C.F & Sharp, M. (1991) ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli*, *Salmonella typhimurium* and other enterobacteria. *Molecular Microbiology*. **5** (4), 825-34.

Huys, G., Janssen, P & Kersters, K. (1997) Is AFLP fingerprinting a true alternative to the DNA- DNA pairing method to assess genospeicies in the genus *Aeromonas*? *International Journal of Systematic Bacteriology*. **47** (1), 245-46.

Imanaka, H., Kousaka, M., Tamura, G & Arima, K. (1965) Studies on pyrrolnitrin, a new antibiotic: taxonomic studies on pyrrolnitrin-prodcuing strain. *Journal of Antibiotics*. **18** (5), 205-7.

Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., Zabeau, M & Kersters, K. (1996) Evaluation of the DNA fingerprinting methods AFLP as a new tool in bacterial taxonomy. *Microbiology*. **142**, 1881-93.

Janssen, P., Maquelin, K., Coopman, R., Tjernberg, I., Bouvet, P., Kersters, K & Dijkshoorn, L. (1997) Discrimination of *Acientobacter* genomic species by AFLP fingerprinting. *International Journal of Systematic Bacteriology*. **47** (4), 1179-87.

Kaper, K.B & Hacker, J (Eds) (1999) Pathogenicity islands and other mobile virulence elements. ASM Press, Washington, U.S.A.

Karaolis, D.K.R., Somara, S., Maneval, J.R., Johnson, J.A & Kaper, J.B (1999) A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. *Nature*. **399**, 375-79.

Kawamoto, S.O & Lorbeer, J.W. (1974) Infection of onion leaves by *Pseudomonas cepacia*. *Phytopathology*. **64**, 1440-45.

Kersters, K., Heulin, T., and Fernandez, M. P. (1995) Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N_2-fixing isolates from rice in Vietnam. *International Journal of Systematic Bacteriology*. **45**, 274-289.

Lacy, D.E., Spencer, D.A., Goldstein, A., Weller, P.H & Daryshire, P (1993) Chronic granulomatous disease presenting in childhood with *Pseudomonas cepacia* septicaemia. *Journal of Infection*. **27**, 301-4.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**, 680-5.

Leigh, M.B., Flecter, J.S., Fu, X & Schmitz, F.J (2002) Root turnover: An important source of microbial substrates in rhizosphere remediation of recalcitrant contaminants. *Environmental Science and Technology*. **36**, 1579-83.

Lessie, T.G., Hendrickson, W., Manning, B.D & Devereux, R (1996) Genomic complexity and plasticity of *Burkholderia cepacia*. *FEMS Microbiology Letters*, **144**, 117-28.

Lewenza, S & Sokol, P.A. (2001) Regulation of ornibactin biosynthesis and *N*-acyl-*L*-homoserine lactone production by CepR in *Burkholderia cepacia*. *Journal of Bacteriology*. **183** (7), 2212-18.

Li, X., Dorsch, M., Del Dot, T., Sly, L.I, Stackebrandt, E & Hayward, A.C. (1993) Phylogenetic studies of the rRNA group II Pseudomonads based on 16s rRNA gene sequences. *Journal of Applied Bacteriology*. **74**, 324-29.

LiPuma, J.J., Dulaney, B.J., McMenamin, J.D., Whitby, P.W., Stull, T.L., Coenye, T & Vandamme, P (1999) Development of rRNA-based PCR assays for identification of *Burkholderia cepacia* complex isolates recovered from cystic fibrosis patients. *Journal of Clinical Microbiology*. **37** (10), 3167-70.

LiPuma, J.J (2000) Minutes from the 5th Annual International *Burkholderia cepacia* Working Group Meeting, Bethesda, Maryland, USA.

LiPuma, J.J., Spilker, T., Coenye, T & Gonzalez, C.F. (2002) An epidemic *Burkholderia cepacia* complex strain identified in soil. *The Lancet*. **359**, 2002-3.

Ludwig, W., Kirchof, G., Klugbauer, N., Weizenegger, M., Betzl, D., Ehrmann, M., Hertel, C., Jilg, S., Tatzel, R., Zitzelsberger, H., Liebl, S., Hochberger, M., Shah, J., Lane, D & Wallnoef, P. R. (1992) Complete 23S ribosomal RNA sequences of Gram-positive bacteria with a low DNA G+C content. *Systematic and Applied Microbiology*. **15**, 487-501.

Magalhães Cruz, L., Maltempi de Souza, E., Baler Webber, O., Baldani, J.I., Döbereiner, J & de Oliveira Pedrosa, F. (2001) 16S ribosomal DNA characterization of nitrogen-fixing bacteria isolated from banana (*Musa* spp.) and pineapple (*Ananas comosus* (L.) Merril). *Applied and Environmental Microbiology*. **67** (5), 2375-79.

Mahenthiralingam, E., Simpson, D.A & Speert, D.P. (1997) Identification and characterization of a novel DNA marker associated with epidemic *Burkholderia cepacia* strains recovered from patients with cystic fibrosis. *Journal of Clinical Microbiology*, **35** (4), 808-16.

Mahenthiralingam, E., Bischof, J., Byrne, S.K., Radomski, C., Davies, J.E., Av-Gay, Y & Vandamme, P (2000a) DNA-based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis*, *Burkholderia multivorans*, *Burkholderia stabilis* and *Burkholderia cepacia* genomovars I and III. *Journal of Clinical Microbiology*. **38** (9), 3165-73.

Mahenthiralingam, E., Coenye, T., Chung, J.W., Speert, D.P., Govan, J.R., Taylor, P & Vandamme, P. (2000b) Diagnostically and experimentally useful panel of strains from the *Burkholderia cepacia* complex. *Journal of Clinical Microbiology*. **38**, 910-13.

Mahenthiralingam, E., Vandamme, P., Campbell, M.E., Henry, D.A., Grarelle, A.M., Wong, L. T., Davidson, A.G., Wilcox, P.G., Nakielna, B & Speert, D.P. (2001) Infection with *Burkholderia cepacia* complex genomovars in patients with cystic fibrosis: virulent transmissible strains of genomovar III can replace *Burkholderia multivorans*. *Clinical Infectious Disease*. **33**, 1469-79.

Mahenthiralingam, E., Bladwin, A & Vandamme, P. (2002) *Burkholderia cepacia* complex infection in patients with cystic fibrosis. *Journal of Medical Microbiology*. **52**, 533-38.

Mao, W., Lewis, J.A., Lumsden, R.D & Hebber, K.P. (1998) Biocontrol of selected soilborne diseases of tomato and pepper plants. *Crop Protection*. **17** (6), 535-42.

Marin, V.A., Teixeira, K.R & Baldani, J.I (2003) Characterization of amplified polymerase chain reaction *gln*B and *Nif*H gene fragments of nitrogen fixing *Burkholderia* species. *Letters in Applied Microbiology*. **36** (2), 77-82.

Marschalek,R., Brechner, T., Amon – Böhm, E & Dingerman, T (1989) Transfer RNA genes: landmarks for integration of mobile genetic elements in *Dictyostelium discoideum*. *Science*. **244**, 1493-6.

Martin, B., Humbert, O., Camara. M, Guenzi, E., Walker, J., Mitchell, P.A., Prudhomme, P., Alloing, G., Hakenbeck, R., Morrison, D.A., Boulnois, G.J & Claverys, J.P. (1992) A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. *Nucleic Acids Research*, **20** (13), 3479-83.

Master, E.R & Mohn, W.W. (2001) Induction of *bphA*, encoding biphenyl dioxygenase, in two polychlorinated biphenyl-degrading bacteria, psychotolerant *Pseudomonas* strain Cam-1 and mesophilic *Burkholderia* strain LB400. *Applied and Environmental Microbiology*. **67** (6), 2669-76.

Matic, I., Taddei, F & Radman, M. (1996) Genetic barriers among bacteria. *Trends in Microbiology*. **4** (2), 69-73.

McGowan, C., Fulthorpe, R., Wright, A & Tiedje, J.M. (1998) Evidence for interspecies gene transfer in the evolution of 2-4-dicholorphenoxyacetic acid degraders. *Applied and Environmental Microbiology*. **64** (10), 4089-92.

McLoughlin, T.J., Quinn, J.P., Bettermann, A & Bookland, R (1992) *Pseudomonas cepacia* suppression of sunflower wilt fungus and role of antifungal compounds in controlling the disease. *Applied and Environmental Microbiology*. **58** (5), 1760-3.

McManus, T.E., Moore, J.E., Crowe, M., Dunbar, K & Elborn, J.S (2003) A comparison of pulmonary exacerbations with single and multiple organisms in patients with cystic fibrosis and chronic *Burkholderia cepacia* infection. *Journal of Infection*. **46**, 56-9.

Mecsas, J & Strauss, J.E (1996) Molecular mechanisms of bacterial virulence: Type III secretion and pathogenicity islands. *Emerging Infectious Diseases*. **2** (4), 271-88.

Miché, L., Faure, D., Blot, M., Cabanne-Giuli, E & Balandreau, J. (2001) Detection and activity of insertion sequences in environmental strains of *Burkholderia*. *Environmental Microbiology*. **3** (12), 766-73.

Miller, S.C.M., LiPuma, J.J and Parke, J.L (2002) Culture-based and non growth-dependent detection of the *Burkholderia cepacia* complex in soil environments. *Applied and Environmental Microbiology*. **68** (8), 3750-58.

Minerdi, D., Fani, R., Gallo, R., Boarino, A & Bonfante, P. (2001) Nitrogen fixation genes in an endosymbiotic *Burkholderia* strain. *Applied and Environmental Microbiology*. **67** (2), 725-32.

Moulin, L., Munive, A., Dreyfus, B & Boivin-Masson, C (2001) Nodulation of legumes by members of the β-subclass of Proteobacteria. *Nature*. **411**, 948-50.

Mueller, J. G., Devereux, R., Santavy, D. L., Lantz, S. E., Willis, S. G., and Pritchard, P. H. (1997) Phylogenetic and physiological comparisons of PAH-degrading bacteria from geographically diverse soils. *Antonie van Leeuwenhoek,* **71**, 329-343.

Nogales, B., Moore, E.R., Llobet-Brossa, E., Rossello-Mora, R., Amann, R & Timmis,K.N. (2001) Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. *Applied Environmental Microbiology*. **67** (4), 1874-1884

O'Callaghan, E.M., Tanner. M.S & Boulnois, G.J. (1994) Development of a PCR probe test for identifying *Pseudomonas aeruginosa* and *Pseudomonas* (*Burkholderia*) *cepacia*. *Journal of Clinical Pathology*. **47**, 222-4

Ouchi, K., Abe, M., Karita, M., Oguri, T., Igari, J & Nakazawa, T. (1995) Analysis of strains of *Burkholderia* (*Pseudomonas*) *cepacia* isolated in a nosocomial outbreak by biochemical and genomic Typing. *Journal of Clinical Microbiology.* **33** (9), 2353-7.

Page, R. D. M. (1996). TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences*. **12**, 357-358.

Page, R, D. M & Holmes, E.C (1998) Chapter 2 Trees. In: Molecular evolution- A phylogenetic approach. Blackwell Science Ltd, Oxford, London.

Palleroni, N.J., Kunisawa, R., Contopoulou, R & Doudoroff, M. (1973) Nucleic acid homologies in the genus Pseudomonas. *International Journal of Systematic Bacteriology*. **23** (4), 333-9

Palleroni, N.J. (1993-94) *Pseudomonas* classification: A new case history in the taxonomy of gram-negative bacteria. *Antonia Van Leeuwenhoek*. **64** (3-4) 231-51

Pan, M.J., Rademan, S., Kunert, K & Hastings, J.W. (1997) Ultrastructural studies on the colonization of banana tissue and *Fusarium oxysporum* f.sp. *cubense* race 4 by the endophytic bacterium *Burkholderia cepacia*. *Journal of Phytopathology*. **145**, 479-86.

Parke, J.L. (1990) Population dynamics of *Pseudomonas cepacia* in the pea spermosphere in relation to biocontrol of *Pythium*. *Phytopathology*. **80** (12), 1307-11.

Parke, J.L. (1998) *Burkholderia cepacia*: friend or foe? *American Phytopathological Society (APS) Net Feature*. http://www.scisoc.org/feature/burkholderacepacia/top.html

Parke, J.L & Gurian-Sherman, D. (2001) Diversity of the *Burkholderia cepacia* complex and implication for risk assessment of biological control strains. *Annual Reviews of Phytopathology*. **39**, 225-58.

Parons, Y.N., Banasko, R., Detsika, M.G., Duangsonk, K., Rainbow, L.C., Hart, A & Winstanley, C. (2003) Suppression-subtractive hybridisation reveals variations in gene distribution amongst the *Burkholderia cepacia* complex, including the presence in some strains of a genomic island containing putative polysaccharide production genes. *Archives of Microbiology*. **179**, 214-23.

Pitt, T.L., Kaufman, M.E., Patel, P.S., Benge, L.C.A., Gaskin, S & Livermore, D.M (1994) Type characterisation and antibiotic susceptibility of *Burkholderia* (*Pseudomonas*) *cepacia* isolates from patients with cystic fibrosis in the United Kingdom and the Republic of Ireland. *Journal of Medical Microbiology*. **44**, 203-10.

Prince, A (1986) Antibiotic resistance of *Pseudomonas* species. *Journal of Pediatrics*. **108** (5), 830-34.

Rademaker, J.L.W., Hoste, B., Louws, F.J., Kersters, K., Swings, J., Vauterin, L., Vauterin, P & deBruijn, F.J. (2000) Comparison of AFLP and rep-PCR genomic fingerprinting with DNA -DNA homology studies: *Xanthomonas* as a model system. *International Journal of Systematic and Evolutionary Microbiology*. **50**, 665-77.

Rahme, L.G., Stevens, E.J., Wolfort, S.F., Shao, S.F., Tompkins, R.G & Ausbel, F.M. (1995) Common virulence factors for bacterial pathogenicity in plants and animals. *Science*. **268**, 1899-1902.

Rainbow, L., Hart, C.A & Winstanley, C (2002) Distribution of type III secretion gene clusters in *Burkholderia pseudomallei*, *B. thailandensis* and *B. mallei*. *Journal of Medical Microbiology*. **51**(5), 374-84.

Rapp, P (2001) Multiphasic kinetics of transformation of 1,2,4- Trichlorobenzene at nano- and micromolar concentration by *Burkholderia* sp strain PS14. *Applied and Environmental Microbiology*. **67** (8), 3496-3500.

Richard, C., Monteil, H., Megraud. F., Chatelain, R & Laurant, B. (1981) Caracteres phenotypiques de 100 souches de *Pseudomonas cepacia* proposition d'un schema de biovars. *Annales de Biologie Clinque*. **39**, 9-15.

Richardson, J., Stead, D.E & Coutts R.H.A (2001) Incidence of the *cblA* major subunit pilin gene amongst *Burkholderia* species. *FEMS Microbiology Letters*. **196**, 61-66.

Richardson, J., Stead, D.E., Elphinstone, J.G & Coutts, R.H.A. (2002) Diversity of *Burkholderia* isolates from woodland rhizosphere environments. *Journal of Applied Microbiology*. **93**, 616-30.

Roca, A.I & Cox, M.M (1990) The *recA* protein structure and function. *Critical Reviews In Biochemistry and Molecular Biology*. **25** (6), 415-456.

Rodley, P.D., Romling, U & Tummler, B. (1995) A physical map of the *Burkholderia cepacia* type strain. *Molecular Microbiology*. **17** (1), 57-67.

Rosenthal, A., Coutelle, O & Craxton, M. (1993) Large-scale production of DNA sequencing templates by microtitre format PCR. *Nucleic Acids Research*. **21** (1), 173-4.

Sajjan, U.S., Sun, I., Goldstein, R & Forstner, J.F. (1995) Cable (*Cbl*) type II pili of cystic fibrosis-associated *Burkholderia* (*Pseudomonas*) *cepacia* : nucleotide sequence of the *CblA* major subunit pilin gene and novel morphology of the assembled appendage fibers. *Journal of Bacteriology*. **177** (4), 1030-1038.

Sajjan, U., Liu, L., Lu, A., Spilker, T., Forstner, J & LiPuma, J.J. (2002) Lack of cable pili expression by *cblA*-containing *Burkholderia cepacia* complex. *Microbiology*. **148**, 3477-84.

Sasser, M. (1990) Identification of bacteria through fatty acid analysis. *In Methods in Phytopathology* ed. Klement, Z., Ruldoph, K & Sands, D.C. pp199-203. Budapest : Academiai Kiado.

Sambrook, J., Fritsch, E.F & Maniatis, T. (1989) *Molecular Cloning*, 2nd Edition. Cold Spring Harbour Laboratory Press. USA.

Schwieger F. & Tebbe, C.C. (2000) Effect of field inoculation with *Sinorhizobium meliloti* L33 on composition of bacterial communities in rhizospheres of a target plant (*Medicago sativa*) and a non-target plant (*Chenopodium album*) – linking of 16S rRNA gene-based single-strand conformation polymorphism community profiles to the diversity of cultivated bacteria. *Applied Environmental Microbiology*. **66** (8) 3356-65.

Seah, S.Y.K., Labbé, G., Kaschabek, S.R., Reifenrath, F., Reineke, W & Eltis, L.D. (2001) Comparative specificities of two evolutionary divergent hydrolases involved in microbial degradation of polychlorinated biphenyls. *Journal of Bacteriology*. **183** (5), 1511-16.

Segonds, C., Heulin, T., Marty, N & Chabanon, G. (1999) Differentiation of *Burkholderia* species by PCR-restriction fragment length polymorphism analysis of the 16S rRNA gene and application to cystic fibrosis isolates. *Journal of Clinical Microbiology*. **37** (7), 2201-08.

Sermswan, R.W., Wongratanacheewin, S., Trakulsomboon, S & Thamlikitkul, V (2001) Ribotyping of *Burkholderia pseudomallei* from clinical and soil isolates in Thailand. *Acta Tropica*. **80**, 237-44.

Schwartz, H.F & Mohan, S.K (1965) Sour skin: in compendium of onion and garlic diseases. APS Press. http://www.scisoc.org/feature/burkholderiacepacia/sourskin.html.

Schwieger, F., and Tebbe, C. C. (2000) Effect of field inoculation with *Sinorhizobium meliloti* L33 on the composition of bacterial communities in rhizospheres of a target plant (*Medicago sativa*) and a non-target plant (*Chenopodium album*)-linking of 16S rRNA gene-based single-strand conformation polymorphism community profiles to the diversity of cultivated bacteria. *Applied and Environmental Microbiology*. **66**, 3556-3565.

Smith, N.C., Hennessy & Stead, D.E. (2001) Repetitive sequence-derived PCR profiling using the BOX-A1R primer for rapid identification of the plant pathogen *Clavibacter michiganensis* subspecies *sepedonicus*. *European Journal of Plant Pathology*. **107**, 739-48.

Sneath, P. H. A., Stevens, M & Sackin, M. J. (1981) Numerical taxonomy of *Pseudomonas* based on published records of substrate utlization. *Antonie van Leeuwenhoek*. **47**, 423-48.

Speert, D.P., Bond, M., Woodman, R.C & Curnutte, J.T. (1994) Infection with *Pseudomonas cepacia* in chronic granulomatous disease: role of nonoxidative killing by neutrophils in host defence. *Journal of Infectious Disease*. **170** (6), 1524-31.

Speert, D.P. (2002) Advances in *Burkholderia cepacia* complex. *Paediatric Respiratory Reviews*. **3**, 230-35.

Springael, D., van Thor, J., Goorissen, H., Ryngaert, A., De Baere, R., Van Hauwe, P., *et al*. (1996) RP4::Mu3A mediated *in vivo* cloning and transfer of a chlorobiphenyl catabolic pathway. *Microbiology*. **142**, 3283-3293.

Springael, D., Ryngaert, A., Merlin, C., Toussaint, A & Mergeay, M. (2001) Occurrence of Tn*4371*-related mobile elements and sequences in (chloro) biphenyl-degrading bacteria. *Applied and Environmental Microbiology*. **67** (1), 42-50.

Stainer, R,Y., Palleroni, N.J & Doudoroff, M. (1966) The aerobic Pseudomonads a taxonomic study. *Journal of General Microbiology*, **43**, 159-271.

Stead, D.E. (1988) Identification of bacteria by computer assisted fatty acid profiling. *Acta Horticulturae*, **225**, 39-46.

Stead, D.E (1992) Grouping of plant pathogenic and some other Pseudomonas spp. by using cellular fatty acid. *International Journal of Systematic Bacteriology*. **42** (3), 281-95.

Story, R.M., Weber, I.T & Steitz, T.A (1992) The structure of the *E. coli recA* protein monomer and polymer. *Nature*. **23**, 318-25.

Sun, L., Jaing, R., Steinbach, S., Holmes, A., Campanelli, C., Forstner, J., Sajjan, U., Tan, Y., Riley, M & Goldstein, R (1995) The emergence of a highly transmissible lineage of *cbl+ Pseudomonas* (*Burkholderia*) *cepacia* causing CF centre epidemics in North America and Britain. *Nature Medicine*. **1**, 661-66.

Tabacchioni, S., visca, P., Chiarini, L., Bevivino, A., DiSerio,C., Francelli, S & Fani, R. (1995) Molecular characterisation of rhizosphere and clinical isolates of *Burkholderia cepacia*. *Research & Microbiology*. **146**, 531-42.

Taplin, D., Basset, D.C.J & Mertz, P.M. (1971) Foot lesions associated with *Pseudomonas cepacia*. *Lancet*. **2** (7724), 568-71.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*. **24**, 4876-4882.

Timmis, K.N & Pieper, D.H. (1999) Bacteria designed for bioremediation. *Trends in Biotechnology*. **17**, 201-4.

Tyler, S.D., Rozel, K.R & Johnson, W.M. (1996) Identification of IS*1356*, a new insertion sequence, and its association with IS*402* in epidemic strains of *Burkholderia*

cepacia infecting cystic fibrosis patients. *Journal of Clinical Microbiology*. **34** (7), 1610-16.

Ulrich, J.M. (1975) Pectic enzymes of *Pseudomonas cepacia* and penetration of polygalacturonase into cells. *Physiological Plant Pathology*. **5**, 37-44

Van Damme, P., Johannes, A. G., Cox, H. C., and Berends, W. (1960) On toxoflavin, the yellow poison of *Pseudomonas cocovenenans*. *Recl Trav Chim Pays-Bas Belgl*. **79,** 255-267.

Vandamme, T., Pot, B., Gillis, M., De Vos, P., Kersters, K & Swings, J. (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiological Reviews*. **60** (2), 407-38.

Vandamme, P., Holmes, B., Vancanneyt, M., Coenye, T., Hoste, B., Coopman, R., Revets, H., Lauwer, S., Gillis, M., Kersters, K & Govan, J.R.W (1997) Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and the proposal of *Burkholderia multivorans* sp. nov. *International Journal of Systematic Bacteriology*. **47** (4), 1188-1200.

Vandamme, O., Mahenthiralingam, E., Holmes, B., Coeyne,T., Hoste, B., De Vos.,P., Henry, D & Speert, D.P. (2000) Identification and population structure of *Burkholderia stabilis* sp. Nov. (formerly *Burkholderia cepacia* genomovar IV). *Journal of Clinical Microbiology*. **38** (3), 1042-47.

Vandamme, P., Goris, J., Chen, W.M., de Vos, P & Willems, A. (2002a) *Burkholderia tuberum* sp. Nov. and *Burkholderia phymatum* sp. nov., nodulate the roots of tropical legumes. *Systematic and Applied Microbiology*. **25**, 507-12.

Vandamme P, Henry D, Coenye T, Nzula S, Vancanneyt M, LiPuma JJ, Speert DP, Govan JR, Mahenthiralingam E. (2002b) *Burkholderia anthina* sp. nov. and *Burkholderia pyrrocinia*, two additional *Burkholderia cepacia* complex bacteria, may confound results of new molecular diagnostic tools. *FEMS Immunology and Medical Microbiology*. **33** (2), 143-9.

Vandamme, P., Holmes, B., Coenye, T., Goris, J., Mahenthiralingam, E., LiPuma, J.J & Govan, J.R (2003) *Burkholderia cenocepacia* sp. nov. – a new twist to an old story. *Research in Microbiology*. **154** (2), 91-6.

Versalovic, J., Schneider, M., deBruijn, F.J., and Lupski, J.R. (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cell Biology*. **5**, 25-40.

Vermis, K., Coenye, T., Mahenthiralingam, E., Nelis, H.J & Vandamme, P (2002) Evaluation of species-specific *recA*-based PCR tests for genomovar level identification within the *Burkholderia cepacia* complex. Journal of Medical Microbiology. **51** (11), 937-40.

Viallard, V., Poirier, I., Cournoyer, B., Haurat, J., Wiebkin, S., Ophel-Keller, K., and Balandreau, J. (1998) *Burkholderia graminis* sp. nov. a rhizospheric *Burkholderia* species and reassessment of (*Pseudomonas*) *phenazinium*, (*Pseudomonas*) *pyrrocinia* and (*Pseudomonas*) *glathei* as *Burkholderia*. *International Journal of Systematic Bacteriology*. **48**, 549-563.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M & Zebeau, M. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research.* **23** (21), 4407-4414.

Wang, X.L., Zheng, Y., Liu, R.M., Li, B.G., Cao, J & Tao, S (2003) Medium scale spatial structures of polycyclic aromatic hydrocarbons in the topsoil of Tianjin area. *Journal of Environmental Science and Health- Part B.* **38** (3), 327-35.

Whiteford, M.I., Wilkinson, J.D., McColl, J.H., Colnlon, F.M., Michie, J.R., Evans, T.J & Paton, J.Y. (1995) Outcome of *Burkholderia* (*Pseudomonas*) *cepacia* colonisation in children with cystic fibrosis following a hospital outbreak. *Thorax.* **50**, 1194-98.

Wigley, P & Burton, N.F. (1999) Genotypic and phenotypic relationships in *Burkholderia cepacia* isolated from cystic fibrosis patients and the environment. *Journal of Applied Microbiology.* **86**, 460-68.

Wigley, P& Burton, N.F. (2000) Multiple chromosomes in *Burkholderia cepacia* and *B. gladioli* and their distribution in clinical and environmental stains of *B. cepacia*. *Journal of Applied Microbiology.* **88**, 914-18.

Winkelstein, J.A., Marino, M.C., Johnston, R.B., Boyle, J., Curnette, J., Gall, G.I., Malech, H.L., Holland, S.M., Ochs, H., Quie, P., Buckley, R.H., Foster, C.B., Chanock, S.J & Dickler, H. (2000) Chronic granulomatous disease. Report on a national registry of 368 patients. *Medicine.* **79** (3), 155-69.

Winstanley, C., Hales, B.A & Hart, C.A. (1999) Evidence for the presence in *Burkholderia pseudomallei* of a type III secretion system associated gene cluster. *Journal of Medical Microbiology.* **48**, 649-56.

Winstanley, C & Hart, C.A (2001) Type III secretion systems and pathogenicity islands. *Journal of Medical Microbiology.* **50** (2), 116-26.

Whitby, P.W., Carter, K.B., Hatter, K.L., LiPuma, J.J & Stull, T.L. (2000) Identification of members of the *Burkholderia cepacia* complex by species-specific PCR. *Journal of Clinical Microbiology.* **38** (8), 2962-65.

Woese, C.R. (1987) Bacterial Evolution. *Microbiological Reviews.* **51** (2), 221-71.

Yabuuchi, E., Kosako,Y., Oyaizu, H., Yano., I., Hotta, H., Hashimoto, Y., Ezaki, T & Arakawa, M. (1992) Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiology & Immunology.* **36** (12), 1251-75.

Yabuuchi, E., Kawamura, Y., Ezaki, T., Ikedo, M., Dejsirilert, S., Fujiwara, N., *et al.* (2000) *Burkholderia uboniae* sp. nov., L-arabinose-assimilating but different from *Burkholderia thailandensis* and *Burkholderia vietnamiensis*. *Microbiology and Immunology.* **44**, 307-317.

Yohalem, D.S & Lorbeer, J.W (1994) Intraspecific metabolic diversity among strains of *Burkholderia cepacia* isolated from decayed onions, soils and the clinical environment. *International Journal of General & Molecular Microbiology.* **65**, 111-31.

Yohalem, D.S & Lorbeer, J.W. (1997) Distribution of *Burkholderia cepacia* phenotypes by niche, method of isolation and pathogenicity to onion. *Annals of Applied Biology*. **130**, 467-9.

Zhang, H., Hanada, S., Shigematsu, T., Shibuya, K., Kamagata, Y., Kanagawa, T., and Kurane, R. (2000) *Burkholderia kururiensis* sp. nov., a trichloroethylene (TCE)-degrading bacterium isolated from an aquifer polluted with TCE. *International Journal of Systematic and Evolutionary Microbiology.* **50**,743-749.